Identifying Dominant Anaerobic Microorganisms for Degradation of Benzene

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ABSTRACT

Like other aromatic hydrocarbons, benzene is a common soil and groundwater contaminant. It is recognized as a human carcinogen. Exposure of benzene can cause serious negative impacts on human health. Benzene is of major concern due to its toxicity and relatively high water solubility. Benzene is easily biodegraded by ubiquitous bacteria with the presence of free oxygen. However, soil and groundwater contamination with petroleum hydrocarbon often results in the development of anaerobic zones. Bioremediation has been considered as an advantageous alternative in terms of fairly low cost, process flexibility, and on-site utility for the treatment of contaminated soil and groundwater. However, benzene is particularly persistent under anaerobic condition even in the enhanced anaerobic biodegradation process. Although studies have shown that benzene biodegradation could occur under several reducing conditions, the in situ activities of anaerobic benzene degradation are generally low. Bioaugmentation rather than biostimulation may be applicable to accelerate biodegradation process. Successful bioaugmentation requires the inoculation of contaminated soil and groundwater with the strains or consortia of specific degrading capabilities. However, information of dominant species within the microorganisms for anaerobic benzene degradation is still limited. To address this problem, in this study, a benzene-degrading nitrate-reducing culture was established with soil contaminated by gasoline. A nitrate-reducing medium with sulphate, phosphate and other inorganic nutrient was employed to enhance anaerobic benzene degradation. BioSep BioTrap coupled with stable isotope probing and other molecular biological methods were used to identify key anaerobic benzene degraders. Members of genus *Dokdonella spp.*, *Pusillimonas spp.*, and *Advenella spp.* were found to be the dominant microorganisms during anaerobic benzene degradation, and were hypothesized to be benzene degrader under nitrate-reducing condition.

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NOMENCLATURE

abcA	Benzene Carboxylase
APS	Adenosine-5′-phosphosulfate Reductase
BTEX	Benzene, Toluene, Ethylbenzene, Xylene,
bssA	Benzylsuccinate Synthase
DGGE	Denaturing Gradient Gel Electrophoresis
DIC	Dissolved Organic Carbon
DO	Dissolved Oxygen
FAME	Fatty Acid Methyl Esters
FID	Flame Ionization Detector
GC	Gas Chromatography
IRMS	Isotope Ratio Mass Spectrometer
LNAPLs	Light Non-aqueous Phase Liquid
mBGL	Meter Below Ground Level
MPN	Most Probable Number
MS	Mass Spectrometry
NAPL	Non-aqueous Phase Liquid
NIR	Nitrite Reductase
ORP	Oxidation-reduction Potential
PAH	Polycyclic Aromatic Hydrocarbon
PHC	Petroleum Hydrocarbon
PHC F1	Volatile Petroleum Hydrocarbons C_6-C_{10}
PHC F2	Volatile Petroleum Hydrocarbons > C_6-C_{10}
PHC F3	Volatile Petroleum Hydrocarbons > C_{16}-C_{34}
PLFA	Phospholipid Fatty Acid
qPCR	Quantitative Polymer Chain Reaction
SIP	Stable Isotope Probing
TP	Total Phosphate

Chapter 1. INTRODUCTION AND OBJECTIVES

1.1. Background

Since the beginning of the industrial revolution, the irrepressible demands for energy to meet global industrialization have largely expanded the frontiers of petroleum hydrocarbon (PHC) exploration. Everyday products ranging from methane to asphalt are produced from different fractions of petroleum. Canada is the third largest producer of natural gas, the fifth largest energy producer, and the sixth largest crude oil producer in the world (Canadian Association of Petroleum Products 2013). In 2012, total Canadian crude oil production reached 3.2 million barrels/day. With such huge amounts of production, it is inevitable that a mass quantity of various PHC components will be introduced into the environment during every stage of oil extraction, refinement, storage, transportation, use, and disposal (Chapelle 1999; Ollivier and Magot 2005; Okoh and Trejo-Hermandez 2010).

Benzene is naturally occurring in petroleum and is particularly found in gasoline. Gasoline contains numerous compounds where the mass fraction of benzene were 0.0076 and 0.0021 for fresh and weathered gasoline, respectively (EPA 1996). Benzene, toluene, ethylbenzene, and o-, m-, and p-xylene isomers (BTEX) were found to account for as much as 90 % of the gasoline components in water soluble parts (Saeed and Mutairi 1999). Compared to other aromatic hydrocarbons, benzene is highly water-soluble (1780 mg/L at 20 ℃). Also benzene is defined as toxic by Canadian Environmental Protection Act and is classified as a Group A human carcinogen by US Environmental Protection Act. With high solubility and toxicity, benzene contamination is of major concern.

Benzene is readily biodegradable under aerobic condition (Gibson 1986; Clark et al. 1990; Harwood and Parales 1996). Aerobic benzene-degrading bacteria are ubiquitous and many microorganisms have been found capable of using benzene as the sole carbon and energy source during biodegradation process.

The most notable microorganisms are the *Pseudomonas* species which account for up to 87 % of the identified aerobic benzene degraders in contaminated aquifers (Coates et al. 2002).

However, aerobic aquifers with organic contamination always transform into anaerobic zones as a result of the combination of the rapid oxygen consumption and slow replenishing rate of oxygen supply by diffusion (Anderson and Lovely 1999). In general, degradation of BTEX compounds under anaerobic condition is more difficult than under aerobic condition. All studies regarding anaerobic BTEX degradation have indicated that anaerobic benzene degradation is most difficult whereas toluene is comparatively easy to degrade (Weelink et al. 2008). Benzene's chemical structure indicates that it is very difficult to initiate enzymatic attack on benzene ring under anaerobic condition. It probably results in the persistence of benzene under anaerobic condition. Anaerobic benzene biodegradation usually occurs very slow, incomplete, and is associated with long lag time (Edwards and Grbic-Galic 1992; Vogt et al. 2011). Some studies suggest that anaerobic benzene degraders are not ubiquitous. It possibly explains why benzene was only slowly degraded or not degraded at all at some sites or in some laboratory enrichment cultures (Kazumi et al. 1997; Nales et al. 1998; Weiner and Lovely 1998; Phelps and Young 1999).

To address this problem, enhanced bioremediation through biostimulation and bioaugmentation is a very attractive option to clean up contaminated sites (Korda et al. 1997; Chapelle 1999). Bioremediation, which relies on microbial activity to decompose organic contaminants, is widely practised for soil and groundwater pollution with organic contaminants. Using organic contaminants as substrates for carbon and energy source, naturally occurring microbes in soil and groundwater are able to convert hydrocarbons and other organic compounds into end products (i.e. carbon dioxide and water). In most cases, biostimulation is important to enhance the ability of indigenous microbes by delivery of appropriate amounts of electron acceptors and its preferable nutrients.

Bioaugmentation is one of the main bioremediation strategies (Speight and Arjoon 2012). It involves inoculating contaminated soil and groundwater with the right strains or consortia of specific contaminant-degrading capabilities. Bioaugmentation can be applied to promote anaerobic benzene biodegradation at those sites that lack microbes responsible for benzene elimination. One of the knowledge gaps that leads to avoidance of bioaugmentation application on anaerobic benzene bioremediation is the lack of information about anaerobic benzene degraders.

1.2. Research Objectives

Benzene is a widely spread contaminant yet difficult to remediate. For successful in situ benzene bioremediation, the overall objective of this research was identifying dominant indigenous benzene-degrading microorganisms under anaerobic condition. Benzene-degrading microorganisms are suspected not ubiquitous or naturally dominant in the environment since repeated attempts to isolate responsible bacteria have failed by other researchers. Therefore, characterization of benzene-degrading consortium could reveal valuable information of microorganisms that mediate degradation process, and enable better strategies of biostimulation and bioaugmentation processes for bioremediation of contaminated sites.

In this manner, the overall objective of this study was divided into two sub objectives:

- To establish optimal anaerobic benzene-degrading culture by altering medium composition through biostimulation;
- To identify dominant microorganisms present in the microcosms using molecular approaches.

1.3. Scope of Study

This study was conducted to identify dominant indigenous benzene-degrading microorganisms under anaerobic condition. Benzene was the only contaminant of interest in this study. As a preliminary test for in situ bioremediation to clean up benzene contamination, lab scale experiments with controlled environment (temperature, nutrients composition etc.) were carried out. The growth of indigenous benzene-degrading microorganisms was stimulated by optimization of media recipes. Contaminated soil and groundwater samples were collected from a sole gasoline contaminated site. Other hydrocarbons originated from soil and groundwater samples were negligible as a result of dilution when introduced into the experiments. Due to analytical methods limitation, bacteria identification was carried out using PCR-DGGE and is only as precise as in genus level.

1.4. Thesis Outline

Chapter 2 provides the theoretical background and an overview of the research conducted in this area. Chapter 3 describes the methodologies to address the two sub-objectives that were to investigate the optimal medium composition for benzene degradation, and to identify anaerobic

benzene degraders by molecular biology approaches. Results of this research and a general discussion are provided in Chapter 4. Finally, conclusions and recommendations of this work are described in Chapter 5.

Chapter 2. LITERATURE REVIEW

2.1. Petroleum Hydrocarbon Contamination

Petroleum is a highly complex and variable mixture. Over a long time, as a result of anaerobic biodegradation and high temperature/pressure, the organic material has been converted to natural gas, liquid crude oil, shale oil, and tars. The majority of the compounds in petroleum are petroleum hydrocarbons ranging from light-molecular-weight gas methane to high-molecular-weight bitumen. These petroleum hydrocarbons can also be divided to straight and branched chains, single or condensed rings, and aromatic rings by molecule structures (Scragg 2004). Two major groups of aromatic hydrocarbons are the monocyclic aromatic hydrocarbons such as BTEX, and the polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, anthracene, and phenanthrene. When crude oil is refined after several procedures, most of the PAHs are converted into monocyclic aromatic hydrocarbons.

At every point in oil production, distribution, consumption, and disposal, oil and its derivatives are inevitably released into the environment, which can pollute both soil and groundwater. Leakage from underground and aboveground storage tanks, improper disposal of the wastes, and accidental spills are recognized as the major sources of soil and groundwater contamination with petroleum products (Nadim et al. 2000). The most common route of soil and groundwater contamination with PHC is leakage from underground storage tanks that are used by gas stations.

PHC constituents may partition into four phases in subsurface: vapor (in soil gas), residual (adsorbed onto soil particles including organic matter), aqueous (dissolved in water), and separate or immiscible (liquid hydrocarbons) (EPA 1996). Liquid hydrocarbons that exist as separate or free phase when in contact with water and/or air are called non-aqueous phase liquids (NAPL). Those hydrocarbon compounds with lower density than that of water are called light non-aqueous phase liquids (LNAPLs). Gasoline and other fuel oil are common LNAPLs (National Research Council 1994).

In general, LNAPLs are potential long-term sources for continued soil and groundwater contamination at many sites. High concentration of BTEX has been detected in soil, sediments, and groundwater around contamination area. BTEX exposure is very harmful to human health and the environment. Benzene is a known human carcinogen with leukaemia potential (Budavari et al. 1989). Toluene and xylene are not carcinogenic; yet have the ability to enhance carcinogenesis by other compounds. Due to their mobility and toxicity, BTEX compounds have been listed as priority pollutants (EPA 1996).

2.2. Remediation of Petroleum Hydrocarbon Contamination

In response to a growing need to address environmental contamination, many remediation technologies have been developed. Based on the process acting on the contaminant, remediation techniques to remove or reduce the effect of a contaminant in the environment can be classified into four categories, which are removal, separation, destruction, and containment (Speight and Arjoon 2012). A combination of biological, physical, and chemical technologies may be used to allow optimum remediation and reduce the contamination to a safe and acceptable level (Khan et al. 2004). Soil remediation technologies include soil vapor extraction, bio-slurry systems, phytoremediation, bioventing, and aeration etc. Common groundwater treatment technologies include air sparing, groundwater pump-and-treat technology, bio-slurping, and natural attenuation etc. Each of these methods may involve some level of risk, and has the recognized drawbacks like incomplete removal and leaving contaminant residual. Depending on the chemical constituents of the spilled contaminant, these physical or chemical methods may be of high cost, and have limited effectiveness (Nadim et al. 2000).

Bioremediation, on the other hand, has been recognized as a promising alternative to control PHC contamination. Bioremediation is a natural process functioning on biodegradation, and can lead to complete mineralization. With the help of naturally occurring bacteria or fungi, bioremediation turns the organic contaminants into harmless final product including carbon dioxide, water, inorganic compounds, and cells biomass (Gibson and Sayler 1992). Bioremediation is cost effective, and in some cases, shows high degrees of effectiveness for the treatment of the PHC contaminated sites. In general, many indigenous microorganisms in soil and groundwater are capable of degrading hydrocarbon contaminants.

The application of bioremediation falls into two broad categories, ex situ bioremediation and in situ bioremediation (Speight and Arjoon 2012). Ex situ bioremediation requires excavation of contaminated soil or pumping contaminated groundwater before treatment, while in situ bioremediation treats the contaminated soil and groundwater in the location where found (Chapelle 1999). Since in situ bioremediation does not require excavation or pumping whereas ex situ bioremediation does, in situ bioremediation is less expensive, causes less release of contaminants during the process, and has the ability to treat large volume at once.

The factors required for the success of bioremediation process include: the presence of metabolically capable and sustainable microbial population, the quantity and quality of oxygen and other electron acceptors, appropriate levels of nutrients (nitrogen, phosphorus, sulfur, and other nutrients to support microbial growth), bioavailability of the contaminants, optimal operating temperature for microbial species growth (most degradation occurs at temperatures between 10 °C and 35 °C), favorable acidity or alkalinity (best range from pH 6.5 to pH 7.5), and the presence of water (Speight and Arjoon 2012).

Biodegradation is the key process in bioremediation. A variety of complex biodegradation patterns emerge from physical interactions between contaminations and soil matrix, and from biological interactions among different microorganisms (Margesin and Schinner 2005). Aerobic biodegradation of PHCs is relatively rapid and complete. Since oxygen consumption is relatively fast and oxygen supply is rather slow due to low oxygen solubility in water, anaerobic zone usually occurs in PHCs contaminated groundwater and soil. Conventional in situ bioremediation of PHC contaminated soil and groundwater relies on the supply of oxygen to enhance aerobic biodegradation process. Yet introducing oxygen into contaminated area can be technically difficult and expensive. Anaerobic bioremediation can be significant in oxygen depleted area.

Anaerobic biodegradation of aromatic hydrocarbons has been identified at field site and in microcosm studies, and then has been demonstrated in lab scale experiment under several electron acceptor conditions (Reinhard et al. 1984; Lee et al. 1987). Bioremediation of PHC contamination can be enhanced to increase the effectiveness and to reduce time required to meet cleanup objectives. Enhanced bioremediation technologies involve the addition of nutrients (biostimulation) or microorganisms (bioaugmentation). Biostimulation is a remediation method which stimulates the growth and reproduction of naturally occurring bacteria by adding nutrients and electron

acceptors that limit indigenous bacteria growth. The success of biostimulation is case-specific, depending on present indigenous microbial population and organic material in the contaminated environments. Bioaugmentation is the addition of pre-grown microbial cultures that are adapted or genetically engineered to enhance microbial population at site to improve contaminant biodegradation rate and reduce clean-up time. Indigenous microbes are sometimes present in very small quantities. Bioaugmentation adds highly concentrated and specialized populations of microorganisms to decontaminate the pollutant of interest at site (Atlas 1991).

2.3. Benzene Properties

Benzene (CAS Number: 71-43-2) is classified as a simple cyclic chemical organic compound (physical and chemical properties of benzene is summarized in Table 2.1). Its molecular formula is C_6H_6, of which the molar mass is 78.11 g/mol. Its density is 0.8765 g/cm^3. Benzene is a non-polar substance with relatively high solubility in water. Benzene is highly volatile with vapor pressure of 94.8 mmHg (25 ˚C). It's a colorless, flammable liquid with strong aromatic odour at room temperature. Benzene is composed and stabilized by an aromatic ring system. It has a continuous pi bond in which six carbon atoms join together in a ring without any potentially reactive substituent. The chemical structure indicates benzene is chemically stable in the environment.

Table 2.1 Physical and chemical properties of benzene. (US EPA)

Property	**Value**
Molar Mass (g/mol)	78.11
Density (g/cm^3)	0.8765
Melting point (°C)	5.5
Boiling point (°C)	80.1
Solubility in water (mg/L)	1791 (25 °C)
Henry's Law constant (atm-m^3/mol)	5.55E-03
Log K_{ow} (L/kg)	2.13
Log K_{oc} (L/kg)	1.77
Vapor Pressure (mm Hg)	94.8 (25 °C)

Benzene is defined as toxic by Canadian Environmental Protection Act (1988). It is determined that benzene constitutes or may constitute a danger in Canada to human life or health.

Benzene is a known human carcinogen and a toxicant without a threshold value (a substance with some probability of harm at any level of exposure). Acute toxicity includes corneal injury to the eye or skin irritant with harmful amounts. Inhalation of benzene can irritate the respiratory tract and may result in central nervous system depression and possible death due to respiratory failure. Ingestion and subsequent aspiration into the lungs may cause chemical pneumonitis. Inhalation LC_{50} for rat is 13,050-14,380 ppm/4H; oral LD_{50} for rat is 1800 mg/kg. Chronic toxicity includes drying and scaling of the skin with prolonged or repeated exposure. Long term exposure has been associated with certain types of leukemia effects on humans. Chronic exposure to benzene has been reported to cause bone marrow abnormalities and adverse blood effects including anaemia. Besides cancer hazard, benzene may cause adverse birth reproductive effects; multiple myelomas, fetotoxicity, teratogenicity have been linked to benzene exposure. The effects of benzene release to the environment pose serious threats to human health. Canadian Drinking Water Guidelines (2009) allows a maximum acceptable concentration of 5 µg/L of benzene in drinking water.

2.4. Aerobic Benzene Biodegradation

Like many other aromatic compounds, benzene is easily and readily biodegradable in the presence of oxygen. Aerobic benzene degradation process has been extensively studied and well understood for a long time (Clark et al. 1990; Harwood and Parales 1996; Gulensoy and Alvarez 1999; Nicholson and Fathepure 2005; Fahy et al. 2006). Aerobic benzene-degrading microorganisms have been found ubiquitous and numerous aerobic benzene degraders have been identified (Damborsky et al. 2000). The key aerobic benzene-degrading microorganisms include *Pseudomonas*, *Comamonas*, *Alcaligenes*, *Acinetobacter*, and *Burkholderia* spp. (Liou et al. 2008).

Benzene can be oxidized to phenol by *Pseudmoonas* pickettii PKO1, and then to hydroquinone by *Nitrosomonas europea* ATCC19718 (Heider and Fuchs 1997). *P.putida* 39/D is able to utilize and oxidize benzene to *cis*-dihydrodiol derivatives (Ziffer et al. 1973). *P.putida* ML2 was found to utilize benzene as the sole carbon and energy source (Zhang and Bouwer 1997). Benzene was also converted by both Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Mycobacterium rhodochrous* to 3, 5-cyclohexadiene-l, 2-diol, then to pyrocatechol through *cis*-muconic acid and 3-oxoadipate to succinate and acetyI-CoA (Cruden et al. 1992; Zhang and Bouwer 1997).

Under aerobic condition, biodegradation of benzene involves initial attack of molecular oxygen resulting in the formation of unstable metabolite *cis*-benzene dihydrodiol (also known as diol). This

hydroxylation reaction is catalyzed by a dioxygenase. *Cis*-benzene is then dehydrogenated to catechol by a dehydrogenase (Jindrova et al. 2002). Ring cleavage, which is to introduce a substituent group on to the benzene ring, occurs through two possible alternative mechanisms. One is monohydroxylation by monooxygenase, and the other is dihydrxolation by dioxygenases.

2.5. Anaerobic Benzene Biodegradation

In aquifer environment, rather small carbon loads in the environment can lead to anaerobic condition owing to low oxygen solubility in water. Since anoxic condition is always established, anaerobic biodegradation of recalcitrant and persistent organic contaminants is of great interest and environmental significance. For alkylated aromatic hydrocarbons, like toluene, xylene, and ethylbenzene, degradation pathways and involved bacteria ecology have been well studied and described (Weelink et al. 2008). However, benzene has long been doubted to be biodegradable without the presence of free oxygen.

Prior to 1980s, there was no evidence of anaerobic benzene degradation. The first described anaerobic benzene degradation was with mixed methanogenic cultures established from sewage (Grbic-Galic and Vogel 1987). A sequence of redox zones were found to be always developed as a result of organic contamination in aquifers (Christensen et al. 2001). Methanogenic conditions were observed near the source of the organic pollutants, where sulfate-reducing and iron-reducing conditions existed in downstream of the plume, and nitrate and manganese-reducing conditions were detected at the fringes of the plume.

While benzene is one of the most persistent compound within PHCs complex, its biodegradability is highly dependent on the environmental conditions. The number of benzene-degrading laboratory microcosms and enrichment cultures has been increased in the last decade. But the number is still low when compared with the cultures described for anaerobic degradation of other aromatic hydrocarbons (i.e. toluene). The rate and extent of benzene degradation are mostly dependent on two factors:

- The quantity and quality of electron acceptors and nutrients;
- The type, number, and metabolic capacities of the microorganisms.

However, establishing efficient anaerobic degradation of benzene has been shown to be difficult. In most cases, anaerobic benzene degradation usually requires long lag time before its actual taking

place and being detectable. Detected biodegradation rate is generally rather low. The reasons for this recalcitrance of benzene are still unknown. Nevertheless, several aspects are speculated:

- Co-contaminants were shown to inhibit anaerobic benzene degradation (Edwards et al. 1992; Cunningham et al. 2001; Ruiz-Aguilar et al. 2003; Silva and Alvarez 2007);
- Several researchers found that anaerobic benzene degradation did not occur at some sites (Barbaro et al. 1992; Kazumi et al. 1997; Nales et al. 1998; Phelps and Young 1999; Morasch et al. 2001). This may indicate that anaerobic benzene degrader is not ubiquitous;
- Syntrophic consortia seem to be required for optimal degradation activity (Phelps et al. 1998; Kunapuli et al. 2007; Vogt et al. 2007; Kleinsteuber et al. 2008; Sakai et al. 2009; Herrmann et al. 2010).

2.5.1. Anaerobic Benzene Degradation under Different Reducing-Conditions

Anaerobic benzene degradation has been demonstrated under several different electron acceptors conditions with laboratory microcosms and enrichment cultures. In most cases, microcosms that were prepared from contaminated soil or sediments usually show shorter lag time and better actual biodegradation activities compared with microcosms that were prepared from contaminated groundwater (Holm et al. 1992).

Table 2.2 provides the summary of the balanced stoichiometric equations and standard free Gibson energy changes for benzene with different electron acceptors allowing bacterial growth. The energy required for microorganisms' cell synthesis and cell maintenance is produced from electrons transferring from electron donors to electron acceptors. Supposedly, the redox gradients for the microbial activities are formed corresponding to the potential energy available by the individual electron acceptor. Interestingly, the growth behavior of anaerobic benzene-degrading cultures seems to not follow this rule. It can be seen that the standard Gibson free energy for nitrate-reducing or ferric iron-reducing reactions are more than 10 times higher than that of sulfate-reducing and carbon dioxide-reducing reactions. Nevertheless, the biomass yields of nitrate-reducing enrichment were reported to be comparable or even lower than the yields of methanogenic-reducing enrichment (Coates et al 2001; Ulrich and Edwards 2003).

Table 2.2 Stoichiometric equations and standard free energy changes for benzene (C_6H_6) oxidations with various electron acceptors with and without biomass ($C_5H_7O_2N$) formation (Weelink 2008).

Electron Acceptor (ox/red)	Stoichiometric Equation (w/,w/o biomass)	ΔG° (kJ/mol)
SO_4^{2-}/H_2S	$C_6H_6 + 3.75SO_4^{2-} + 3H_2O \rightarrow 6HCO_3^- + 1.875H_2S + 1.875HS^- + 0.375H^+$	-186
	$C_6H_6 + 3.44SO_4^{2-} + 2.63H_2O + 0.12NH_4^+ \rightarrow 5.38HCO_3^- + 1.72H_2S + 1.72HS^- + 0.12C_5H_7O_2N + 0.34H^+$	
NO_3^-/NO_2^-	$C_6H_6 + 15NO_3^- + 3H_2O \rightarrow 6HCO_3^- + 15NO_2^- + 6H^+$	-2,061
	$C_6H_6 + 7.76NO_3^- + 0.83H_2O + 0.72NH_4^+ \rightarrow 2.38HCO_3^- + 7.76NO_2^- + 0.72C_5H_7O_2N + 3.11H^+$	
NO_3^-/N_2	$C_6H_6 + 6NO_3^- \rightarrow 6HCO_3^- + 3N_2$	-2,978
	$C_6H_6 + 2.52NO_3^- + 0.87NH_4^+ \rightarrow 1.65HCO_3^- + 1.26N_2 + 0.87C_5H_7O_2N + 0.87H_2O$	
Fe^{3+}/Fe^{2+}	$C_6H_6 + 30Fe^{3+} + 18H_2O \rightarrow 6HCO_3^- + 30Fe^{2+} + 36H^+$	-3,040
	$C_6H_6 + 12.41Fe^{3+} + 6.57H_2O + 0.72NH_4^+ \rightarrow 1.6HCO_3^- + 12.41Fe^{2+} + 0.88C_5H_7O_2N + 14.9H^+$	
ClO_3^-/Cl^-	$C_6H_6 + 5ClO_3^- + 3H_2O \rightarrow 6HCO_3^- + 5Cl^- + 6H^+$	-3,813
	$C_6H_6 + 1.83ClO_3^- + 0.13H_2O + 0.14NH_4^+ \rightarrow 1.21HCO_3^- + 1.81Cl^- + 0.96C_5H_7O_2N + 2.17H^+$	
CO_2/CH_4	$C_6H_6 + 6.75H_2O \rightarrow 2.25HCO_3^- + 3.75CH_4 + 2.25H^+$	-124
	$C_6H_6 + 6.3H_2O + 0.08NH_4^+ \rightarrow 2.04HCO_3^- + 3.75CH_4 + +0.08C_5H_7O_2N + 2.13H^+$	

- **Anaerobic benzene degradation under nitrate-reducing condition**

Nitrate is a preferable alternative to oxygen in many ways (Hutchins and Wilson 1994; Wilson and Bouwer 1997). It is cheap, and highly soluble in water without harming other aquifer microorganisms below the concentration of 500 mg/L. Most of all, nitrate-reducing bacteria are ubiquitous and widely distributed in the environment (Caldwell et al. 1999).

Anaerobic benzene degradation under nitrate-reducing condition has been demonstrated in microcosms (Major et al. 1988; Nales et al. 1998), in enrichment cultures (Burland and Edwards 1999; Ulrich and Edwards 2003; Mancini et al. 2008), and in pure cultures (Coates et al. 2001; Kasai et al. 2006).

The first demonstrated anaerobic benzene degradation using nitrate as electron acceptor was conducted by Major et al. (1988). The batch microcosms were from shallow sand aquifer, and anoxic groundwater. Benzene, toluene, and the isomers of xylene decreased with the addition of nitrate, and vice versa. Accumulation of nitrogen gas during the process indicated that nitrate was produced to nitrogen gas.

A microcosm survey was conducted to determine anaerobic benzene biodegradation activity under a variety of electron acceptors conditions (Nales et al. 1998). The microcosms were prepared with saturated soil from several hydrocarbon contaminated sites or with anoxic groundwater and sediments. The defined mineral medium was prepared as described (Edwards et al. 1992). After one year of incubation, anaerobic benzene degradation was observed under sulfate-reducing, nitrate-reducing, and iron-reducing condition, but not under methanogenic condition. Toluene, ethylbenzene, and xylene were found to competitively inhibit benzene degradation under all conditions.

Complete anaerobic benzene mineralization was observed in enrichment cultures established from soil and groundwater mixture (Burland and Edward 1999). It was observed that 92-95 % of amended [^{14}C] benzene was recovered as $^{14}CO_2$. There was also an increase in cell protein over time, which indicated that the anaerobic benzene oxidation yielded energy to support cell growth. Nitrate was reduced incompletely to nitrite, rather than its complete reduction to nitrogen gas. After being incubated in laboratory for several years, the dominant phylotypes in the culture were determined by 16S rRNA genes library, and were found to belong to *Betaproteobacteria* (93 %

similar to genera *Azoarcus* and *Dechloromonas*) (Ulrich and Edwards 2003). The results indicate the importance of *Betaproteobacteria* in anaerobic benzene degradation under nitrate-reducing conditions.

To date only four benzene-degrading pure strains have been isolated. Strain RCB was isolated from river sediments with 4-chlorobenzoate as electron donor and chlorate as electron acceptor. Strain JJ was isolated from lake sediments with a humic-substance analogue as electron donor and nitrate as electron acceptor. *Dechloromonas* strain RCB and strain JJ are phylogenetically closely related with 98.1 % 16S rRNA sequence similarity. Both strains were able to degrade benzene (160 µM) completely to CO_2 coupled to the reduction of nitrate within 5 days (Coates et al. 2001). Strain RCB was found to degrade benzene and toluene concurrently under nitrate-reducing condition. Besides nitrate, strain RCB could degrade benzene coupled with perchlorate, chlorate, and oxygen (Chakraborty et al. 2005). However, metabolic analysis of this strain failed to support anaerobic benzene degradation by missing central enzymes to form universal central intermediate benzoyl-CoA (Salinero et al. 2009).

Strain AN9 and Strain DN11 are affiliated to *Azoarcus* genus (Kasai et al. 2006). Those two strains were isolated using a non-selective medium out of gasoline contaminated groundwater incubated with [^{14}C] benzene. Strain DN11 could grow on benzene, toluene, xylene, and benzoate as electron donor and nitrate as electron acceptor. Strain DN11 could significantly enhance the anaerobic benzene degradation rate after addition of the strain to lab batches indicating its great potential for bioaugmentation application (Kasai et al. 2007).

- **Anaerobic benzene degradation under sulfate-reducing condition**

Anaerobic benzene degradation under sulfate-reducing condition has been found in a column study (Vogt et al. 2007), in microcosms (Edwards and Grbic-Galic 1992; Lovley et al. 1995; Phelps et al. 1996; Coates et al. 1996; Kazumi et al. 1997; Nales et al. 1998; Phelps et al. 1998; Weiner and Lovley 1998; Anderson and Lovley 2000), and in enrichment (Phelps et al. 1998; Caldwell and Suflita 2000; Ulrich and Edwards 2003; Vogt et al. 2007; Herrmann et al. 2008; Kleinsteuber et al. 2008; Mancini et al. 2008; Musat and Widdel 2008; Oka et al. 2008; Berlendis et al. 2010).

Edwards and Grbic-Galic (1992) found that benzene was completely mineralized to carbon dioxide coupled with sulfate reduction. The microcosm was prepared with gasoline contaminated subsurface sediments collected from Seal Beach (California). Later in marine harbour (San Diego

Bay, California) sediments microcosms, Lovley et al. (1995) found that [^{14}C] benzene was completely oxidized to $^{14}CO_2$. It was estimated that sulphate reduction contributed to approximately 80 % benzene degradation in the sediments.

Enrichment cultures from marine sediments were established and could mineralize benzene while using sulfate as the terminal electron acceptor (Phelps et al. 1996). The cultures used sulfidogenic medium as described by Widdel (1980). Repeated attempts to isolate key microorganisms had failed. Phelps et al. (1998) then used molecular approaches such as traditional cloning and sequencing and a direct PCR fingerprinting method to characterize this benzene-degrading sulfate-reducing consortium. Despite the culture's long exposure to benzene as sole carbon and energy source over 3 years, this consortium has remained relatively complex indicating a syntrophic process governing anaerobic benzene degradation within this culture. Four clones (SB-9, 21, 29, and 30) fell within the family *Desulfobacteraceae*, the other phylotypes were affiliated to *Thiomicrospira*, *Sulfurovum*, *Bellilinea*, *Exiguobacterium*, and several other members of the *Clostridia* and *Bacteroidetes* (Wang et al. 2007). Phylotypes related to *Desulfobacteraceae* were also detected to be dominant in several benzene-degrading sulfate-reducing freshwater enrichments (Ulrich and Edwards 2003; Mancini et al. 2008; Musat and Widdel 2008; Oka et al. 2008). These collective results suggest that members of *Desulfobacteraceae* are key microorganisms of benzene degradation under sulfate-reducing condition.

Field and lab DNA-based stable isotope probing (DNA-SIP) experiments showed that dominant sequences in sediments microcosms incubated with [^{13}C] benzene were related to *Pelomonas* (Liou et al. 2008). A member of the *Cryptanaerobacter/Pelotomaculum* group within the gram-positive family *Peptococcaceae*, and a phylotype related to the genus of the *Epsilonproteobacteria* showed significant increase in a benzene-degrading sulfate-reducing laboratory enrichment culture after repeated spike of benzene (Vogt et al. 2007; Kleinsteuber et al. 2008; Abu Laban et al. 2009). Further molecular analysis revealed that the relative abundance of the terminal restriction fragments of these two bacteria increased greatly in heavy fractions of [^{13}C] benzene incubated microcosms compared to controls supplied with [^{12}C] benzene (Herrmann et al. 2010). Based on the possible functions of community members and thermodynamics calculations, a syntrophic association for benzene mineralization was proposed, where *Cryptanaerobacter/ Pelotomaculum* were responsible for initial steps of benzene degradation and the release of reduced

metabolites such as hydrogen or other low molecular weight fermentation products usable for a syntrophic partner (Kleinsteuber et al. 2008; Herrmann et al. 2010).

- **Anaerobic benzene degradation under iron-reducing condition**

Anaerobic benzene degradation under iron-reducing condition has been found in microcosms (Lovley et al. 1994, 1996; Anderson et al. 1998; Anderson and Lovley 1999; Botton and Parsons 2006), and in enrichment cultures (Rooney-Varga et al. 1999; Botton and Parsons 2007; Kunapuli et al. 2007).

The first evidence of complete mineralization of benzene coupled to reduction of ferric iron came from studies by Lovley et al. (1994) with aromatic hydrocarbons contaminated aquifer sediments. It was reported that the addition of organic ligands that bind to Fe(III) dramatically increased insoluble Fe(III) oxides bioavailability, and thus increased degradation rate of aromatic hydrocarbons. Iron-dependent benzene mineralization could be stimulated by Fe(III) being chelated to such compounds as EDTA, N-methyliminodiacetic acid, ethanol diglycine, humic acids, and phosphates (Lovley et al. 1996; Caldwell et al. 1999).

One of the best-investigated iron-driven aquifers took place in Bemidji site (Minnesota, USA) and the investigation lasted over a quarter century (Baedecker et al. 1993; Cozzarelli et al. 1994; Anderson et al. 1998; Rooney-Varga et al. 1999; Cozzarelli et al. 2010; Essaid et al. 2011). Studies at Bemidji site were among the first to document the importance of anaerobic biodegradation processes for hydrocarbon removal and remediation by natural attenuation. Anaerobic benzene degradation and mineralization with sediments and groundwater from this site were repeatedly reported.

Recently, benzene degradation was verified directly by an in situ microcosms approach (Cozzarelli et al. 2010). Investigation of community structure using MPN-PCR revealed an increase of *Geobacter*-related 16S rRNA gene copies, indicating that *Geobacteraceae* is involved in anaerobic benzene degradation under iron-reducing condition. *Geobacteraceae* was also found dominant in an iron-reducing benzene-degrading culture originated from a landfill in Netherland (Botton and Parsons 2006). Under iron-reducing condition, *Geobacteraceae* species were often observed in anaerobic aromatic compounds.

However, Kunapuli et al. (2007) employed [^{13}C] benzene DNA-based stable isotope probing experiment in highly enriched iron-reducing culture from a contaminated site in Poland and found no *Geobacteraceae*. A phylotype related to *Peptococcaceae* (genus *Pelotomaculum*), *Desulfobulbaceae*, and *Actinobacteria* was found prominent in the culture. A syntrophic process was suspected where members of the *Peptococcaceae* appeared to be responsible for the initial attack on benzene ring and assimilation of carbon from [^{13}C] benzene during anaerobic benzene decomposition. Recently, a pure culture of a hyperthermophilic archaeon called *Ferroglobus placidus* capable of mineralizing benzene under iron-reducing condition has been studied (Holmes et al. 2011).

- **Anaerobic benzene degradation under methanogenic condition**

Anaerobic benzene degradation under methanogenic condition has been found in microcosms (Kazumi et al. 1997; Weiner and Lovley 1998), and in enrichments (Vogel and Grbic-Galic 1986; Grbic-Galic and Vogel 1987; Ulrich and Edwards 2003; Chang et al. 2005)

Methanogenic enrichment cultures were initially established from sewage sludge and pre-enriched with ferulic acid as substrate, and benzene was found to be partially mineralized (Vogel and Grbic-Galic 1986; Grbic-Galic and Vogel 1987). But the fate of benzene was not determined, and no data was provided to demonstrate that methanogenesis was the predominant process.

Kazumi et al. (1997) discovered the production of significant quantities of methane following the addition of benzene to aquifer sediments slurry. Subsequent experiment with [^{14}C] benzene found that [^{14}C] benzene was converted to $^{14}CH_4$ and $^{14}CO_2$. Complete mineralization of benzene to carbon dioxide and methane was shown by Weiner and Lovley (1998). The microcosms were developed from heavily gasoline-contaminated aquifer sediments. [^{14}C] benzene was converted to $^{14}CH_4$ and $^{14}CO_2$.

Ulrich and Edwards (2003) developed several enrichments from different contaminated sites under nitrate, sulfate, or methanogenic conditions. While the highest concentration of substrate utilization and maximum degradation rate of benzene was observed with methanogenic enrichment culture. Notably, two cultures initially enriched with sulfate-reducing condition could switch to carbon dioxide and vice versa. This finding supports the hypothesis of syntrophic process governing anaerobic benzene degradation. Phylotypes affiliated to the genera *Desulfobacterium*

(OR-M2) and *Desulfosporosinus* (OR-M1) as well as aceticlastic methanogens were identified as dominant microorganisms (Ulrich and Edwards 2003; Silva and Alvarez 2007; Mancini et al. 2008).

More recently, Sakai et al. (2009) employed DNA-based stable isotope probing and identified Hasda-A within class *Deltaproteobacteria* as one of the key benzene degraders in the enriched methanogenic culture established from non-contaminated lotus field soil. The bacterium was found to be incorporated with the majority of [^{13}C] benzene. The study concluded that syntrophic relation existed in which benzene was degraded by fermenters, aceticlastic methanogens, and hydrogentrophic methanogens together.

2.5.2. Anaerobic Benzene Degradation Pathway

Benzene is chemically stable due to its symmetric π-electron system that lacks potential reactive substituents. The question how benzene is activated without the presence of oxygen is still not convincingly answered. Three putative degradation pathways have been proposed for the initial step in degradation of benzene under anaerobic condition, which are hydroxylation of benzene yielding phenol, methylation of benzene yielding toluene, and carboxylation of benzene yielding benzoate (Figure *2.1*). After the initial activation of the benzene ring, subsequent transformation of benzene was to universal aromatic intermediate benzyl-CoA and ring cleavage.

Each of the three activation reactions has its own advantages and drawbacks, which will be discussed in detail in the following sub sections. Due to lack of pure bacterial strain, most studies to elucidate the reaction mechanisms were using enrichment cultures. Generally, isotope-based methods were performed either by compound specific stable or by detection of stable isotope labelled metabolites.

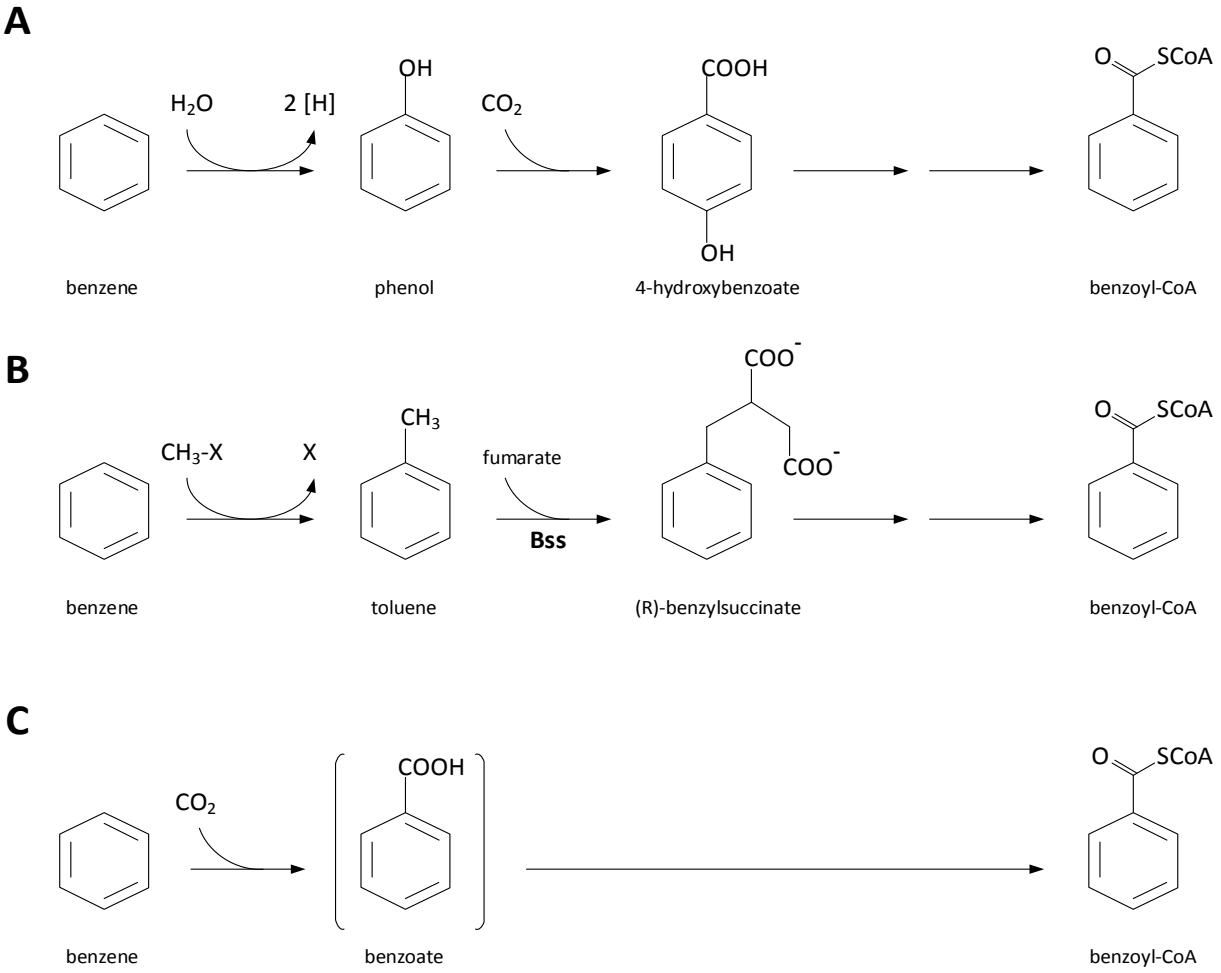

Figure 2.1 Possible mechanisms of anaerobic benzene degradation initial steps (Weelink 2008)

- **Benzene Hydroxylation**

In one of the earliest studies of anaerobic benzene degradation, phenol, cyclohexanone, and propanoic acid were found as intermediates in a methanogenic enrichment culture (Vogel and Grbic-Galic 1986). This leads to a direct hydroxylation as its initial reaction of benzene activation reaction. It was proposed that benzene degradation involved hydroxylation of benzene to phenol followed by the subsequent reduction of phenol to cyclohexanone, then cleaved to aliphatic acids. Subsequent experiment with [^{18}O] water resulted in [^{18}O] phenol suggested that the introduced hydroxyl group incorporated into the benzene ring was derived from water. Weiner and Lovley (1998) also reported that phenol, propionate, and acetate were produced in methanogenic cultures with [^{14}C] benzene experiment.

Dechloromonas strain RCB initially hydroxylated benzene to phenol, and subsequently carboxylated to benzoate in a nitrate-reducing benzene-degrading enrichment (Chakraborty and Coates 2005). When cells degraded benzene in $H_2{}^{18}O$ enriched medium, the formation of [^{18}O] phenol was only slightly accumulated. This indicated hydroxyl group did not originate from water. They hypothesized that hydroxylation of benzene was rather mediated through hydroxyl free radical formed on the outer membrane or in the periplasm of strain RCB. In a following study, Chakraborty et al. (2005) suggested a single universal pathway for anaerobic benzene activation reaction in strain RCB because phenol and benzoate have been detected in several other studies under different electron acceptors conditions.

Phenol and benzoate were concomitantly observed as putative benzene intermediates in sulfate-reducing enrichment, methanogenic cultures, and iron-reducing consortia (Caldwell and Suflita 2000; Ulrich et al. 2005; Botton and Parsons 2007). The hydroxylation of benzene occurred either through incorporation of hydroxyl group from water, or through hydroxyl free radicals onto the benzene ring to form phenol. The degradation of phenol proceeds via a carbon dioxide dependent carboxylation of the aromatic ring to 4-hydrxobenzoate and further transformation to benzoyl-CoA.

Recently Kunapuli et al. (2007) and Abu Laban et al. (2009) found that phenol can be abiotically formed due to the exposure of air during sampling. It was also shown in substrate tests with two sulfate-reducing enrichments where phenol was either consumed immediately (Abu Laban et al. 2009), or after a certain lag time (Musat and Widdel 2008). The results strongly

suggested that phenol was not an intermediate during benzene degradation in the culture. Therefore, one must be careful when interpret phenol as a metabolite during benzene degradation.

- **Benzene Methylation**

Biologically mediated alkylation of benzene to toluene and alkylation of toluene to xylene was observed with bone marrow (Flesher and Myers 1991). The reaction required unique biological methyl donors S-adenosyl-methionine. Alkylation of benzene to toluene was energetically favorable using S-adenosyl-methionine or methyl-tetrahydrofolate as the methyl donor (Coates et al. 2002).

The first direct evidence of benzene methylation was found by Ulrich et al. (2005). In nitrate-reducing and methanogenic enrichment cultures, [$^{13}C_6$] benzene was used as substrate. [^{13}C] toluene and [^{13}C] benzoate were detected as metabolites. This observation supported methylation of benzene as the initial activation reaction of benzene ring, and degradation via the benzylsuccinate synthase pathway was hypothesized. When benzene is methylated, the produced toluene could be further mineralized to the methyl group of toluene catalysed by enzyme benzylsuccinate synthase. Anaerobic toluene degradation has long been known and well researched (Heider 2007). The unique activation mechanism for anaerobic toluene degradation is by addition of fumarate to the methyl group of toluene through benzylsuccinate synthase.

However, some highly enriched nitrate-reducing cultures cannot degrade toluene probably excludes the hypothesis that toluene might be an intermediate during benzene degradation in those cultures (Kunapuli et al. 2008; Musat and Widdel 2008; Abu Laban et al. 2009).

- **Benzene Carboxylation**

Carboxylation of benzene to form benzoate was first detected in a sulfate-reducing culture by Caldwell and Suflita (2000). [^{13}C] benzoate was formed coupled with the spike of [^{13}C] benzene, showing that the carboxyl group of benzoate was from benzene itself instead of from the non-labelled bicarbonate buffer system. Phelps et al. (1996) found that the addition of [^{14}C] bicarbonate to an enriched sulfate-reducing benzene-degrading marine culture did not lead to accumulation of ^{14}C incorporation into the carboxyl group of benzoate. This was in accordance with the result presented by Caldwell and Suflita (2000). It is speculated that the transformation of benzene to benzoate was a much more complex process than simple carboxylation through addition of carbonate.

In contrast, Kunapuli et al. (2008) found both [$^{13}C_6$] benzoate and [$^{13}C_7$] benzoate using [$^{13}C_6$] benzene as substrate in an iron-reducing enrichment. Additional experiment with medium composed of non-labelled benzene and [^{13}C] bicarbonate buffer was conducted. [^{13}C] carboxyl group of benzoate was formed. This result supports the hypothesis that the bicarbonate buffer was the carboxyl group donor for benzoate in this culture.

Benzoate was also identified as a metabolite in a sulfate-reducing culture, providing further evidence for the carboxylation of benzene (Abu Laban et al. 2009). Subcultures were grown on benzene, phenol, or benzoate as sole carbon source, and peptide sequences were identified based on the metagenome that had been sequenced earlier by Kunapuli (2008). Based on the result, benzene carboxylation catalyzed by a putative benzene carboxylase gene was suggested (Abu Laban et al. 2010). The formed benzoate was further mineralized by a benzoate-CoA ligase to benzoyl-CoA.

Holmes et al. (2011) reported accumulation of benzoate in the pure cultures of hyperthermophilic archaeon *Ferroglobus placidus* in an iron-reducing benzene-degrading culture. Analysis of gene transcript levels of this culture revealed an increase in the expression of gene Ferp_1630 that encode enzymes of anaerobic benzoate degradation in the cells grown on benzene versus those grown on acetate. A putative benzene carboxylase gene similar to the one identified by Abu Laban et al. (2010) was highly expressed in cells supplied with benzene compared to those fed with benzoate. These results supported carboxylation of benzene to benzoate as the activation mechanism of benzene ring.

However, it is difficult to interpret all these findings since benzoate (or benzoyl-CoA) is acted as a central intermediate for all putative pathways. As a matter of fact, benzoyl-CoA is a universal intermediate within the anaerobic degradation pathways for most aromatic compounds utilization (Fuchs et al. 2011).

2.5.3. Anaerobic Benzene Degraders

Traditionally, investigations relied on enrichment cultures to identify and isolate microbes responsible for ecologically significant biochemical processes (Madsen 2005). Since anaerobic benzene-degraders are not ubiquitous or naturally abundant, researchers turned to molecular biology techniques, such as denaturing gradient gel electrophoresis (DGGE) and stable isotope probing (SIP) for identification of microbes capable of degrading benzene under anaerobic

condition (Kasai et al. 2006; Kunapuli et al. 2007; Liou et al. 2008; Sakai et al. 2009; Herrmann et al. 2010). A survey of anaerobic benzene-degrading cultures revealing anaerobic benzene degrader using molecular biology methods are summarized (Table 2.3). Bacteria related to families of *Peptococcaceae*, *Geobacteraceae*, and *Desulfobacteraceae* have been found in iron-reducing, sulfate-reducing, and methanogenic benzene-degrading cultures (Phelps et al. 1998; Rooney-Varga et al. 1999; Ulrich and Edwards 2003; Chang et al. 2005; Da Silva and Alvarez 2007; Kunapuli et al. 2007; Kleinsteuber et al. 2008; Musat and Widdel 2008; Oka et al. 2008; Holmes et al. 2011; Van der Zaan et al. 2012). The only anaerobic benzene-degrading isolates related to the genera *Dechloromonas* and *Azoarcus* have been obtained under nitrate-reducing condition (Coates et al. 2001; Chakraborty and Coates 2005; Kasai et al. 2006; Kasai et al. 2007).

Table 2.3 Microorganisms in enriched or pure cultures that were involved in anaerobic benzene degradation.

Electron acceptor	Source of inoculum	Organism, Phylogeny	References
Nitrate-reducing	Sediments from the Potomac River, Maryland, USA	*Dechloromonas strain RCB (Betaproteobacteria)*	Coates et al. 2001; Salinero et al. 2009
	Sediments from Campus Lake, Southern Illinois University, USA	*Dechloromonas* strain JJ (*Betaproteobacteria*)	
	Groundwater from BTX-contaminated subsurface aquifer, Kumamoto, Japan	*Azoarcus* strain DN11	Kasai et al. 2006, 2007
		Azoarcus strain AN9	
	Soil from benzene contaminated industrial location, northern part of the Netherlands,	*Peptococcaceae*	Van der Zaan et al. 2012
Sulfate-reducing	Sediments from an area of deep-water hydrocarbon seeps in the Guaymas Basin, Gulf of California, Mexico	*Desulfobacteraceae* Clone SB-21	Phelps et al. 1996, 1998; Oka et al. 2008
	Sediments from a stagnant part of a Mediterranean lagoon, Etang de Berre, France	*Desulfobacterium* Clone BznS295 (*Deltaproteobacteria*)	Musat and Widdel 2008
	Groundwater from a former coal hydrogenation and benzene production plant, Zeitz, Saxonia-Anhalt, Germany	*Cryptanaerobacter/ Pelotomaculum (Peptococcaceae*)	Kleinsteuber et al. 2008; Herrmann et al. 2010
	Soil from a former coal gasification site in Gliwice, Poland	*Pelotomaculum*	Abu Laban et al. 2009
	Groundwater from 100 km west of Paris, France	*Pelobacter; Desulfobacca*	Berlendis et al. 2010
Iron-reducing	Sediments from the USGS Groundwater Toxics Site, Bemidji, MN, USA	*Geobacter (Geobacteraceae)*	Anderson et al. 1998; Rooney-Varga et al. 1999
	Soil from a former coal gasification site, Gliwice, Poland	*Clostridia (Peptococcaceae); Deltaproteobacteria (Desulfobulbaceae)*	Kunapuli et al. 2007; Abu Laban et al. 2010
	Hydrothermally heated marine sediments, Vulcano, Italy	*Ferroglobus placidus*	Holmes et al. 2011
Methanogenic	Soil and groundwater from a decommissioned retail gasoline station on Cartwright Avenue, Toronto, Canada	*Desulfuromonadales* Clone OR-M2 *(Desulfobacterium)*	Ulrich and Edwards 2003; Silva and Alvarez 2007
	Sediments from Baltimore Harbor, Baltimore, MD, USA	*Bacteroidetes, Euryarchaeota, Firmicutes*, and *Thermotogae*	Chang et al. 2005
	Sediments from a coal-tar waste-contaminated site, Glen Falls, NY, USA	*Pelomonas (Betaproteobacteria)*	Liou et al. 2008
	Soil from Lotus field, river sediments, and industrial waste dumping site, Tsuchiura, Ibaraki, Japan	*Deltaproteobacteria* Clone Hasda-A	Sakai et al. 2009

2.6. Syntrophic Mineralization of Benzene under Anaerobic Condition

Syntrophy is defined as a tightly coupled mutualistic interaction with a minimal number of intermediates for efficient cooperation among the partners (Sieber et al. 2012). The metabolic nature of syntrophy and the limited number of available cultures make it difficult to characterize syntrophic metabolism and identify key metabolic players within complex microbial communities for anaerobic benzene degradation under different conditions.

Stable isotope probing (SIP) has helped to identify key microorganisms involved in transformation of substrates of interest, and to link specific metabolic activity to these microorganisms (Dumont and Murrell 2005). Compounds labelled with stable isotopes (^{13}C, ^{15}N, ^{18}O) are provided as substrates. The incorporation of the stable isotope-labelled tracer is revealed by phospholipid fatty acids (PLFA), nucleic acids, or amino acids. These compounds can either be separated by gradient centrifugation (in case of nucleic acids DNA and RNA), or analyzed by isotope ratio mass spectrometry (in case of PLFA and amino acids) (Radajewski et al. 2000; Neufeld et al. 2007; Jehmlich et al. 2008; Jehmlich et al. 2010). Nowadays, SIP is mostly applied during laboratory microcosm cultures and enrichments.

A syntrophic community degrading benzene under denitrifying conditions during an eight-year chemostat study was demonstrated by DNA-SIP with [^{13}C] benzene (Van der Zaan et al. 2012). Bacteria belonging to the *Peptococcaceae* were identified as dominant benzene degraders, also those related to *Rhodocyclaceae* and *Burkholderiaceae* were found to be associated with the anaerobic benzene degradation process.

In sulfate-reducing microcosm, benzene was degraded through syntrophic association between members of *Peptococcaceae*, *Epsilonproteobacteria*, and *Deltaproteobacteria* (Kleinsteuber et al. 2008; Herrmann et al. 2010). It was suggested that *Peptococcaceae* was involved in the initial attack on benzene ring, and converting it to hydrogen, acetate, or low molecular weight fermentation products. The role of *Delta-* and *Epsilonproteobacteria* was proposed to be consumption of intermediates such as hydrogen and acetate produced by primary benzene, which reduces hydrogen and acetate concentrations and makes degradation of benzene feasible (Kleinsteuber et al. 2008). Recently, a sulfate-reducing benzene-degrading culture was enriched from an underground gas storage aquifer (Berlendis et al. 2010). The abundant phylotypes were

distantly related to genus *Pelobacter*, *thermotogales*, and *Methanolobus*, indicating that benzene is syntrophically degraded.

In an iron-reducing enrichment culture, genus *Peptococcaceae* and *Desulfobulbaceae*-related bacteria were identified as key bacteria involved in degradation of benzene through DNA-SIP (Kunapuli et al. 2007). A syntrophic mode of interaction between these phylotypes was proposed, where *Peptococcaceae* seemed to be responsible for the initial attack on benzene, and *Desulfobulbaceae* appeared to be thriving on the hydrogen produced by *Peptococcaceae*. The relationship between these two microorganisms could be based on hydrogen transfer. In this interaction, hydrogen, which is released by the primary degrader, is utilized by syntrophic partner and therefore pulls the initial reaction towards completion (Kunapuli et al. 2007).

Under methanogenic condition, syntrophic interactions likely exist in all benzene-degrading cultures, as methanogens are not known for degrading aromatic compounds (Vogt et al. 2011). Sakai et al. (2009) proposed that benzene was sequentially degraded by a consortium of fermenters, aceticlastic methanogens, and hydrogentrophic methanogens.

2.7. Review Summary

The monoaromatic hydrocarbons benzene, toluene, ethylbenzene, and the xylenes, are major sources of soil and groundwater contamination with PHCs. Benzene is particularly of great concern due to its carcinogenicity and fairly high water solubility. Benzene is easily degraded under aerobic condition, and is mostly degraded by a large number of aerobic Pseudomonas species. However, in contaminated soil and groundwater area, oxygen is depleted fast leading to extensive anaerobic condition.

Despite its environmental importance, little is known about the microorganisms involved in the anaerobic benzene degradation. The number of laboratory enrichment cultures capable of degrading benzene under anaerobic condition has increased over the last decade. But when compared with the number of cultures described for anaerobic degradation of other aromatic hydrocarbons (e.g. toluene), it is still very low. It is primarily due to the fact that active benzene-degrading cultures are difficult to obtain and maintain. The lack of capable laboratory cultures and the slow growth of the available cultures seem to limit the findings of anaerobic benzene degradation. Maintaining benzene-degrading cultures has been challenging due to problems like the presence of significant lag time, extremely slow degradation rate, or stalling for unknown reasons. Also the diversity of microbes identified in anaerobic benzene-degrading cultures makes attribution of functional microorganisms challenging (Phelps et al. 1998; Ulrich and Edwards 2003; Berlendis et al. 2010).

As a result, this study focuses on identifying key microorganisms involved in anaerobic degradation of benzene. Accomplishing this objective will make a significant progress in the area of anaerobic benzene mineralization.

Chapter 3. METHODOLOGY

In order to fulfill the proposed objective which was identifying key indigenous microorganisms involved in anaerobic degradation of benzene, two main steps were carried out. The initial step was to prepare benzene-degrading culture through biostimulation. The project site was selected through environmental site assessments. Contaminated soil and groundwater samples were collected from the selected site. Several media recipes were tested to determine the optimum growth condition for indigenous benzene-degrades. Once biostimulated benzene-degrading culture was established, the next step was to characterize the benzene-degrading culture and in particular identify dominant microorganisms within the culture. BioSep BioTrap sampler coupled with stable isotope probing was used to understand benzene biodegradation potential within the culture. BioSep BioTrap is proved to be a modern and effective approach for microbial sampling. Post deployment analyses provided evidences from microbiology (e.g. PLFA, SIP, and qPCR) to chemistry (e.g. contaminant loss and DIC) for benzene biodegradation mineralization process. Denaturing gradient gel electrophoresis (DGGE) was then used to identify the dominant members of the microbial community. The procedures are shown in Figure 3.1.

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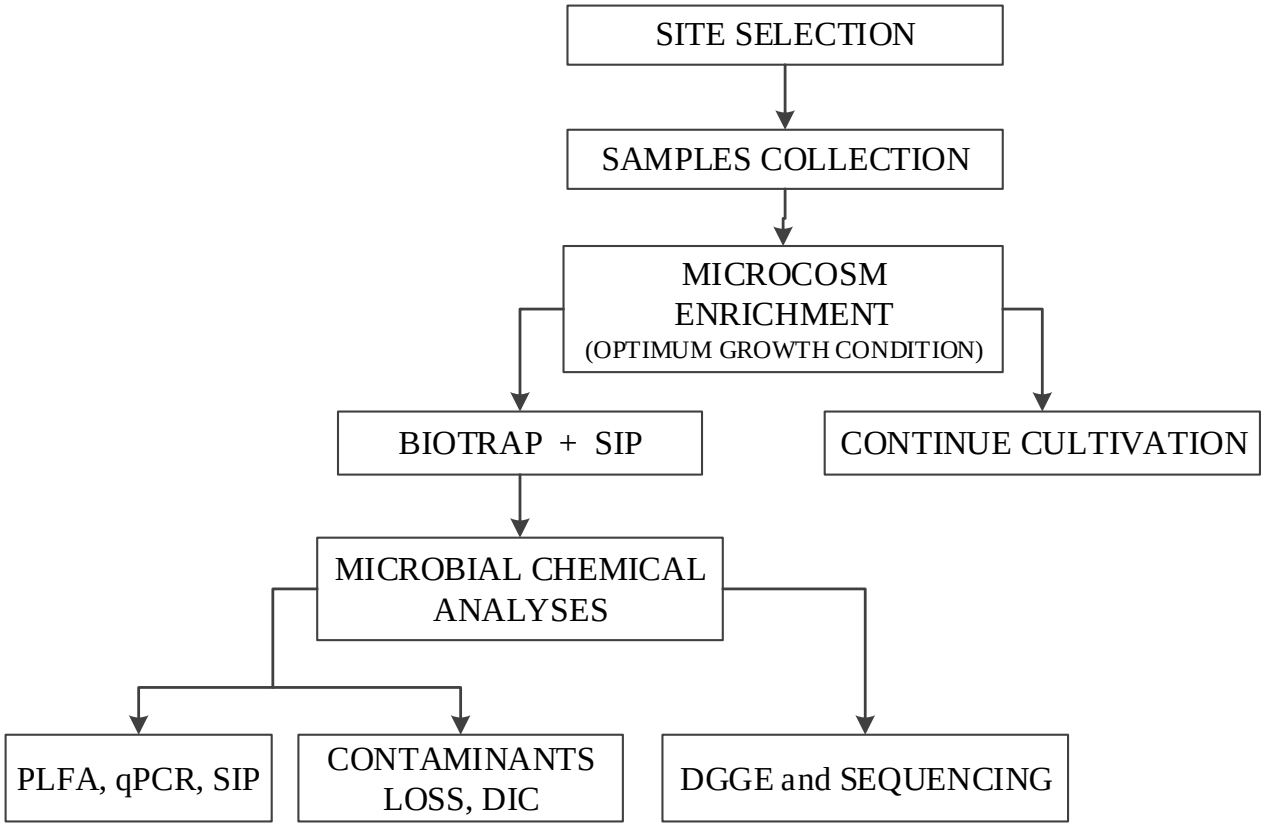

Figure 3.1 Experiment Design Flow Chart

3.1. Site Description

The project site (as shown in Figure 3.2) was a fuel service station for over fifty years. It is situated in Saskatoon, Saskatchewan. The service station formerly contained three underground storage tanks (USTs) to the north of the current existing building and six pump islands to the northeast of the building. Based on Phase II environmental site assessment (ESA), the local stratigraphy beneath the project site consisted of gravel (fill) (to a depth of 1.4 m below ground level (mBGL)), moist and high plastic silty clay (to a depth of 4.3 mBGL), and a stiff medium plastic clay till. The groundwater depth was around 1.4 to 2.0 m from ground level. The groundwater flowed towards the south and southeast.

Boreholes and groundwater monitoring/recovery wells were installed at the project site (as presented in Figure 3.2). A total of 13 boreholes (10-01 to 10-13) were installed at depths between 4.3 to 9.1 m. The soil analytical results of PHCs showed benzene concentration exceeded the

referenced criteria level (2.5 µg/g) in 6 out of 13 boreholes. The maximum concentration of 50 µg/g benzene occurred in borehole 10-09. The rest ranged from 2.97 µg/g to 32 µg/g. Toluene, ethyl benzene, and xylenes were below the reference criteria level in all 13 boreholes. PHC F1 fraction (volatile petroleum hydrocarbons C_6-C_{10}) exceeded the reference criteria level in 4 out of 13 boreholes, with maximum concentration of 9680 µg/g occurred in borehole 10-09. PHC F2 fraction (extractable petroleum hydrocarbons >C_{10}-C_{16}) exceeded the reference criteria level only in borehole 10-09 with concentration of 2990 µg/g. PHC F3 fraction (extractable petroleum hydrocarbons >C_{16}-C_{34}) and PHC F4 fraction (extractable petroleum hydrocarbons C_{34+}) were below the referenced criteria level in all 13 boreholes.

The Phase II ESA groundwater analytical results identified high benzene concentration in boreholes 10-07, 10-08, 10-09, 10-11, and 10-13. High sulfate concentration ranging from 163 mg/L to 2390 mg/L was observed in selective monitor wells (boreholes 10-02, 10-04, 10-09, and 10-11). In all boreholes, ammonia nitrogen was ranging from 0.21 mg/L to 0.4 mg/L; nitrate nitrogen and nitrite nitrogen were beyond detection limit; total phosphorus was ranging from 0.16 mg/L to 0.23 mg/L.

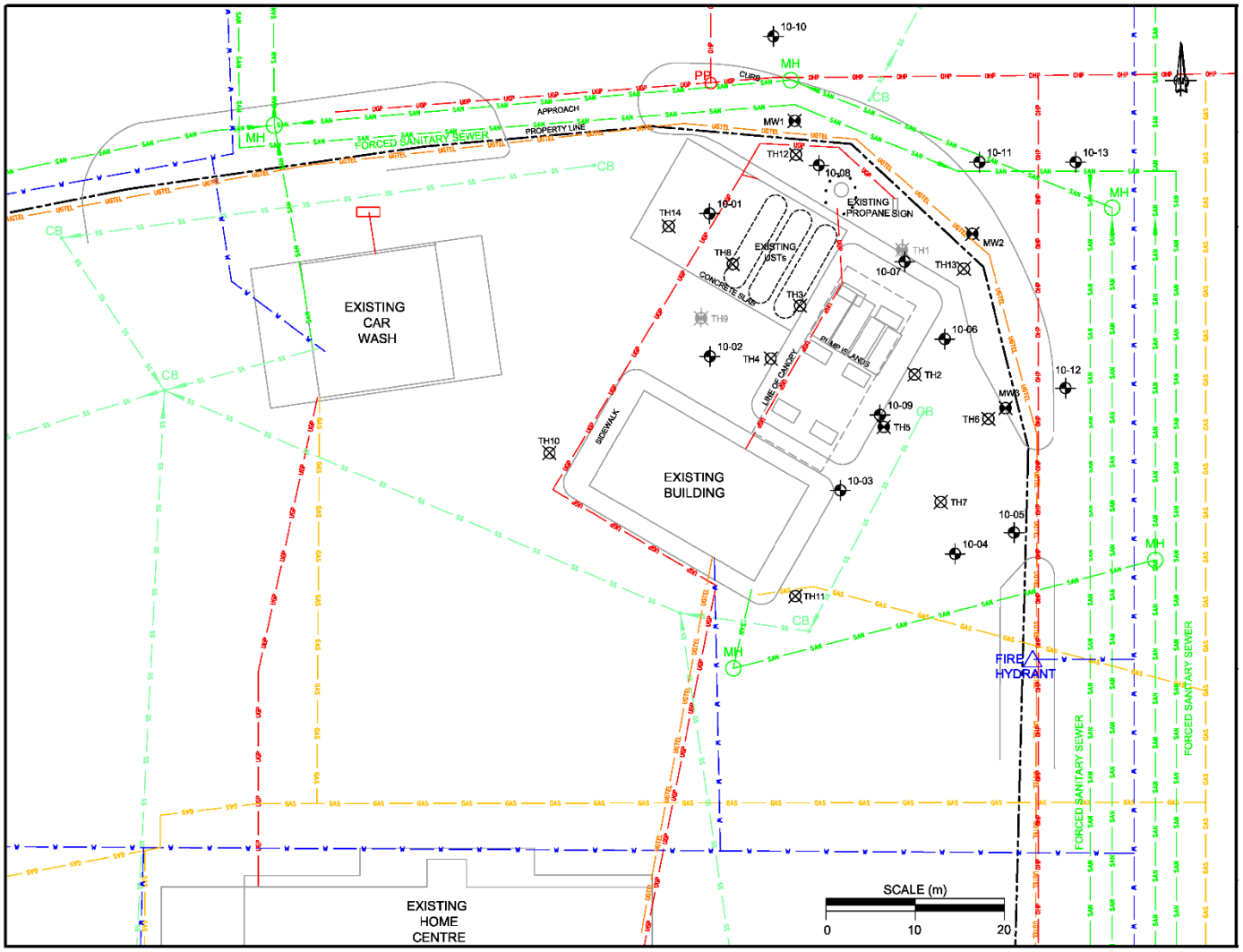

Figure 3.2 A site plan showing the project site with surrounding land use (AMEC Inc. 2010)

3.2. Soil and Groundwater Sampling

Soil samples were collected directly using soil excavation from the project site. The collected soil samples were stored in pails with minimal headspace. Soil samples presented black staining and a strong hydrocarbon odour.

Groundwater samples were collected from groundwater monitoring well (borehole 10-09) using a QED Environmental Systems Micropurge® (MP50) water quality monitoring system with a flow cell at a low flow rate of 0.5 L/min. According to previous ESA analytical results, groundwater within borehole 10-09 had the highest concentration of BTEX and sulfate. Water quality parameters were allowed to stabilize prior to sampling from the purge stream. Groundwater quality parameters including temperature, pH value, electrical conductivity, oxidation-reduction potential (ORP), and dissolved oxygen (DO) were measured immediately after sampling.

The soil and groundwater samples were collected in separate non-reactive containers with minimal headspace, and transported directly back to University of Saskatchewan Environmental Laboratory. The samples were stored at 4 ℃ in lab until use.

3.3. Laboratory Microcosm Inoculum Experiment

In this research, since benzene is the only contaminant of interest, the overall experiment was designed to encourage the growth of benzene-degrading microbes and thus increase benzene degradation rate. The laboratory microcosms were established by benzene contaminated soil or groundwater. Selective media where benzene served as the sole carbon source were employed. Several media recipes adapted from literature review were tested to compare benzene-degrading performance (Coates et al. 1996, 2001; Kazumi et al. 1997; Kasai et al. 2006; Xiong et al. 2012) (Table 3.1).

All materials and chemicals used in this study were of highest available analytical purity and purchased from Fisher-Scientific and Aldrich-Sigma. Soil and groundwater samples were manipulated in an artificial oxygen-free bag filled with high-grade pure nitrogen (99 %) gas. Cultures with Recipe NP were composed of 10 mL groundwater sample and Recipe NP media solution. Cultures with Recipe Coates and Recipe Kazumi were incubated with 10 g soil sample and Recipe Coates and Recipe Kazumi media solution, respectively. The soil slurries consisted of 10 g contaminated soil and 1000 mL media added to sterile 1 L media bottle. Recipes SA, Recipe SA+T, and Recipe SA+T were set up with 100 g soil sample and media solution. The soil slurries

were constructed in the same manner as for Recipe Coates and Recipe Kazumi except that 100 g contaminated soil were used. The soil sample was prepared homogeneously by sieving soil particles through a 2-4 mm sieve. Electron acceptors and inorganic nutrients were supplied to the cultures from sterile anaerobic stock solutions. All media solutions were prepared using standard anaerobic techniques (autoclaved for 30 minute at 121 ℃ twice). Benzene was supplied to the cultures from a neat benzene stock. Ingredients of different recipes and concentration of benzene and inorganic nutrients introduced to the cultures are summarized in Table 3.1.

All recipe media included 15 mL trace element solution and 10 mL vitamin stock. The trace element solution was outlined by Kazumi et al. (1997). Each liter of trace element solution stock contained 30 mg of $CoCl_2 \cdot 6H_2O$, 0.15 mg of $CuCl_2$, 5.7 mg of H_3BO_3, 20 mg of $MnCl_2 \cdot 4H_2O$, 2.5 mg of $Na_2MoO_4 \cdot 2H_2O$, 1.5 mg of $NiCl_2 \cdot 2H_2O$, and 2.1 mg of $ZnCl_2$. The vitamin supplement was purchased from ATCC® MD-VS™. The formulation is based on Wolfe's Vitamin solution, and contains per litter 2 mg folic acid, 10 mg pyridoxine hydrochloride, 5 mg riboflavin, 2 mg biotin, 5 mg thiamin, 5 mg nicotinic acid, 5 mg calcium pantothenate, 0.1 mg vitamin B12, 5 mg p-Aminobenzoic acid, 5 mg thioctic acid, and 900 mg monopotassium phosphate.

All cultures were maintained in 1000 mL Kimax™ GL 45 media/storage bottles with screw-thread opening. The cap was a butyl rubber stopper with Teflon-coated inner side. All bottles were mixed completely and closed tightly. Duplicates were used. Sterile controls were used to rule out abiotic loss due to adsorption and volatilization and thus to demonstrate the biological activity and biodegradation process. Soil and groundwater sterilization was achieved by autoclaving the soil containing experimental bottles 45 min each at 121 ℃ for three times. Abiotic controls were prepared by omitting soil or groundwater from the experimental bottles. Cultures were refilled with sterile anoxic stock media solution after each sampling to avoid headspace. All incubations of microbial cultures were maintained statically without shaking in the dark at room temperature.

Table 3.1 Recipe compositions and introduced benzene concentration.

Recipe	Laboratory Microcosms Set-up	Media Compositions (per liter)	Benzene Concentration (mg/L)
NP	10 mL groundwater	3.5 g $NaNO_3$, 0.3 g K_2HPO_4, 1.49 mg $FeCl_2 \cdot 4H_2O$	25
Coates	10 g soil	4.0 g Na_2SO_4, 1.36 g sodium acetate, 20 g NaCl, 0.5 g KCl, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 0.15 g $CaCl_2 \cdot 2H_2O$, 3 g $MgCl_2 \cdot H_2O$, 1.49 mg $FeCl_2 \cdot 4H_2O$	25
Kazumi	10 g soil	2.84 g Na_2SO_4, 1.3 g KCl, 0.2 g KH_2PO_4, 23 g NaCl, 0.5 g NH_4Cl, 0.1 g $CaCl_2 \cdot 2H_2O$, 1 g $MgCl_2 \cdot H_2O$, 2.5 g $NaHCO_3$, 1.49 mg $FeCl_2 \cdot 4H_2O$	17
SA	100 g soil	2.7 g K_2SO_4, 0.5 g KNO_3, 1 g KH_2PO_4, 0.5 g NH_4Cl, 0.14 g $CaCl_2.2H_2O$, 1 g $MgCl_2.6H_2O$, 2.5 g $NaHCO_3$, 2 mg $FeCl_2.4H_2O$	17
SA+T	100 g soil	10 mg toluene 2.7 g K_2SO_4, 0.5 g KNO_3, 1 g KH_2PO_4, 0.5 g NH_4Cl, 0.14 g $CaCl_2.2H_2O$, 1 g $MgCl_2.6H_2O$, 2.5 g $NaHCO_3$, 2 mg $FeCl_2.4H_2O$	17
SA+P	100 g soil	25 mg benzoate, 35 mg phenol 2.7 g K_2SO_4, 0.5 g KNO_3, 1 g KH_2PO_4, 0.5 g NH_4Cl, 0.14 g $CaCl_2.2H_2O$, 1 g $MgCl_2.6H_2O$, 2.5 g $NaHCO_3$, 2 mg $FeCl_2.4H_2O$	17

3.4. BioTrap Setup

The BioTrap was installed immediately after received to minimize contamination. The BioSep BioTrap used for this study was purchased from a commercial lab named Microbial Insight (Rockford, TN). BioTrap was made of perforated Teflon tubes filled with BioSep beads. The BioSep beads were employed as a substrate and pre-amended with [^{13}C] benzene. BioSep beads are 3 to 4 mm in diameter, and contain 25 % Nomex and 75 % powdered activated carbon. The beads have porous structure with 74 % porosity and internal surface area of 600 m^2/g. The beads are surrounded by ultrafiltration-like membrane with 1-10 micron holes so that nutrients can easily penetrate through. With features like high internal surface area, low shear conditions within the beads, and easy access to limiting nutrients, bacteria are allowed to enter into the beads and rapidly form BioTrap biofilms with bioavailable adsorbed hydrocarbon during incubation.

The microcosm culture where BioTrap was employed was composed of previous incubated microcosm from recipe SA+T. The culture was mixed well before half volume was transformed to a new 1 L sterile experiment bottle. [^{12}C] benzene and [^{12}C] toluene were also introduced into the experimental bottle at concentration of 17 mg/L and 5 mg/L. The bottle was filled to top with sterile anoxic Recipe SA+T media solution to avoid headspace. The BioTrap was mixed completely with surrounding environment, and was submerged in the bottom. After a period of 60 days, the BioTrap was recovered and transferred back to Microbial Insight in a cooler overnight.

3.5. Analytical Methods

Concentrations of benzene and toluene (if applicable) were measured over time. Benzene and toluene were measured by headspace analysis using a gas chromatography (Agilent 7890) equipped with a flame ionization detector (FID) at 300 ℃ connected to a 30 m×0.53 mm×3 μm film DB-624 column and a split/splitless injector (120 ℃) with helium as the carrier gas. The flow rate of the carrier gas is 1 mL min^{-1} and the split ratio was split:splitless=1:10. The oven temperature is programmed to 50 ℃ for 1 min, raised to 200 ℃ at 15 ℃ min^{-1}, and held for 1 minute.

Inorganic nutrients were measured consistently by standard methods. Nitrate concentration was analyzed using Automated Cadmium Reduction Method (4500-NO_3^- F., APHA 1992). A continuous flow analysis was used through an AutoAnalyzer (Technicon, 1973). Sulphate concentration was analyzed using Gravimetric Method with Drying of Reside (4500-SO_4^{2-} D., APHA 1992).

Ammonia concentration was analyzed using Titrimetric Method (4500-NH_3 E., APHA 1992). An Auto Titrator (Metrohm) was used to titrate the pH values for the samples. Total phosphate (TP) concentration was analyzed using Stannous Chloride Method (4500-P D., APHA 1992). A DR/4000U spectrophotometer (Hach, USA) was used to analyze TP.

All the following analysis regarding SIP and other biological analysis were performed by Microbial Insight.

[^{13}C] Benzene concentration within the BioSep beads was determined by extraction with methylene chloride with sufficient time. Analysis was performed using Agilent 6890N GC/ 5973 MSD using an electron ionization source. The temperature was maintained at 230 ℃. Experiment data were collected in SIM mode with a 30 ms dwell time using ions (m/Z) of 78 [^{12}C] and 84 [^{13}C]. Concentrations of [^{13}C] benzene in BioTrap are reported in this study as the mean ± standard deviation (n=3).

Lipids were extracted from BioTrap BioSep beads with a single-phase, chloroform-methanol buffer system (Bligh and Dyer 1954). The total lipid fraction was extracted by silicic acid column chromatography, and the polar lipid fraction was fractionated by alkaline methanolysis to fatty acid methyl esters (FAMEs). The FAMEs were analyzed using a GC/ FID (i.e. Hewlett Packard 5890 series). The separation was accomplished with a 60 m Restek Rtx-1 column, which has 0.25 mm ID and 0.25 μm film thickness. The condition was described before (Pinkart et al. 2002). The GC/MS confirmation of PLFA profiles was accomplished by a Hewlett Packard 5890 GC coupled to a HP5972 quadrupole mass selective detector. The operating conditions were the same as above except a 13-min solvent delay.

The FAMEs derived from extracted phospholipids were measured for [^{13}C] incorporation using a Thermo GC-IRMS system. The system was composed of a Trace Ultra gas chromatograph (Thermo Electron Corp. Milan, Italy) coupled to a Delta Plus Advantage isotope ratio mass spectrometer (IRMS) through a GC/C III interface (Thermo Electron Corp. Bremen, Germany). FAMEs dissolved in hexane were injected in splitless mode and separated on a J&W DB-5 column (30 m × 0.25 mm ID × 0.25 μm film thickness). Once separated, FAMEs were quantitatively converted to CO_2 in an oxidation reactor at 950 ℃. Following water removal through a nafion dryer, CO_2 enters the IRMS for δ^{13}C determination. The δ^{13}C values were corrected using working standards composed of several FAMEs calibrated against NIST standard reference materials.

[^{13}C] isotope incorporation by dissolved inorganic carbon (DIC) was measured by a GasBench II system interfaced to a Delta V Plus IRMS (Thermo Scientific, Bermen, Germany). Water samples (1 to 4 mL) were injected into evacuated 12 mL septum capped vials (Exetainers, Labco, High Wycombe, UK) containing 1 mL 85 % phosphoric acid, which forced the equilibrium between CO_2 and H_2CO_3 to gaseous CO_2. Samples and standards were co-equilibrated before analysis. The evolved CO_2 was purged from vials through a double-needle sampler into a 20 mL/min helium carrier stream. The CO_2 was then passed to the IRMS through a Poroplot Q GC column (15 m × 0.53 mm ID, 25 ℃, 3 mL/min). A reference CO_2 peak was used to calculate provisional $\delta^{13}C$ of the sample CO_2 peak. Final $\delta^{13}C$ values were obtained after adjusting the provisional. Two laboratory standards were analyzed with every 10 samples. The laboratory standards were lithium carbonate (calibrated against NIST 8545) dissolved in de-ionized water.

Real-time quantitative polymer chain reaction (qPCR) was performed on samples using oligonucleotides that were designed to target bssA, abcA, APS, nirS, and nirK. Quantitative PCR quantification was performed on an ABI 7300 Real- time PCR System using PCR primers and Taqman probe. Primers used to detect APS gene targeting SRB were developed by Microbial Insights (Friedrich 2002). Primers used to detect nirS and nirK targeting denitrifying bacteria were described before (Braker et al. 1998). Primers used to detect bssA and abcA targeting anaerobic toluene degrader and anaerobic benzene degrader respectively were used as previously described (Winderl et al. 2007; Abu Laban et al. 2010).

The PCR conditions were as follows: 2 min at 50 ℃ and 10 min at 95 ℃, followed by 50 cycles of 15 s at 95 ℃ and 1 min at 58 ℃. The PCR reaction was carried out in an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). All procedures including annealing and polymerization temperatures, primer concentrations, and $MgCl_2$ concentrations for qPCR were done following the procedure of the manufactures. A calibration curve was obtained by using a serial dilution of a known concentration of positive control DNA. The C_T values that are obtained from each sample are then compared with the standard curve to determine the original sample DNA concentration. All qPCR experiments included appropriate negative and positive control reactions. No amplification was detected in negative controls.

Total bacterial DNA was extracted from BioTrap (approximately 10 to 20 beads) with a PowerSoil extraction kit (MoBio, Inc., Carlsbad, Ca, USA) following the manufactures

recommended protocol. The V3 region of 16S rDNA was amplified with the primers sets 341FGC (GC-clamped oligonucleotide) and 907R (3, 5) with modifications. Thermocycling consisted of 10 m at 94 ℃, then 34 cycles of 94 ℃ for 30 s, 60 ℃ for 45 s and 72 ℃ for 45 s, followed by a 5 m extension at 72 ℃. Each PCR reaction contained 1.25 units of Clontech Advantage 2 polymerase (BD Biosciences) and 10 pmol of each primer listed above in a total volume of 25 µl. The amplification was performed using a Robocycler™ PCR block (Stratagene, La Jolla, CA, USA). This product was then subjected to DGGE using a D-code 16/16 cm gel system (BioRad Lab, USA) maintained at a constant temperature of 60 ℃ in 0.5 TAE buffer (20mM Tris-acetate, 0.5mM EDTA, pH 8.0). Gradients were formed using 8-10 % acrylamide and 30-65 % denaturant (100 % denaturant is defined as 7M urea plus 40 % formamide in 1×TAE buffer) and the gels were run at 55 V for 16 h. after electrophoresis, the gels were stained in 0.5 TAE containing 0.5 mg/l of ethidium bromide, and then put in a shaker at 150 rpm for 10 min followed by excision of the bands from the gel with a pipette tip under UV illumination. Images were captured by the use of an Alpha Imager™ system (Alpha Innotech, San Leandro, USA). Dominant bands were excised, eluted in 50 µL of nanopure DNase/RNase-free sterile water and placed at -20 ℃ for overnight and reamplified using the same primers and conditions listed above. The resulting amplicons were again electrophoresed on a DGGE gel to verify the position of the original band. Subsequently, an Ultra Clean PCR Clean-up DNA Purification Kit (MoBio Laboratories, USA) was used to purify the amplicons following the manufactures directions. The PCR products were then closed using 341F as the primer by the University of Tennessee, Molecular Biology Resource Facility. Chromatogram files that were received from the sequencing facility were then aligned to known DNA sequences using the Ribosomal Database Project. Any base pair mismatches were verified with the original chromatogram or changed based upon that chromatogram. The corrected sequence was then submitted into the same database and the top ten matches were received.

Chapter 4. RESULTS AND DISCUSSION

4.1. Soil and Groundwater Samples

Physical and chemical characteristics of contaminated soil and groundwater obtained for this study were determined and summarized in Table 4.1 and Table 4.2.

Table 4.1 Characteristics of contaminated soil (AMEC Inc. 2010)

Parameters	**Value**
Benzene (mg/kg)	50
Toluene (mg/kg)	10.3
Ethyl Benzene (mg/kg)	251
Xylenes (mg/kg)	1080
PHC F1 (mg/kg)	9680
PHC F2 (mg/kg)	2990
PHC F3 (mg/kg)	120

Table 4.2 Characteristics of contaminated groundwater (AMEC Inc. 2010)

Parameters	**Value**
Temperature (°C)	11.13
pH	6.59
Electrical Conductivity (µs/cm)	8.13
ORP (mV)	−40
DO (mg/L)	0.56
Nitrate (mg/L)	1.24
Phosphate (mg/L)	0.02
Sulphate (mg/L)	14.8
Benzene (mg/L)	10.7
Toluene (mg/L)	1.12
Ethyl Benzene (mg/L)	3.18
Xylenes (mg/L)	3.1
PHC F1 (mg/L)	15
PHC F2 (mg/L)	4.72
PHC F3 (mg/L)	0.051

Since the project site has been contaminated by PHC and high concentration of benzene for decades, the indigenous microorganisms have been exposed to benzene long enough for possible potential adaption to degrade PHC contaminants including benzene.

4.2. Benzene Degradation Using Different Recipes

The overall experiment was designed to encourage growth of microbes capable of degrading benzene rather than compare the recipes themselves. Since microorganisms need organic substances and inorganic nutrients to support cell growth and maintain active bacterial population, appropriate concentration and ratios of nutrients need to be selected to achieve high-level growth of benzene-degrading microorganisms and thus accelerate benzene degradation rate. Direct evidence of benzene biodegradation was based on disappearance of benzene.

4.2.1. Recipe NP

The initial experiment was with Recipe NP and contaminated groundwater. Benzene was only slightly degraded in microcosms within incubation time of 50 days (Table 4.3; Figure 4.1). #NP1 and #NP2 represented two duplicate experimental group results. To evaluate benzene disappearance due to biodegradation, reduced value were determined taking in dilution factor. This same calculation principle was applied to determine other recipes' performance. Slightly less benzene in sterile control and abiotic control can be explained by volatilization during incubation or loss through other means. Degradation percentage over 50 days was 13.20 % and 11.02 % for #NP1 and #NP2 respectively. Degradation percentage was calculated by the difference between initial benzene concentration and final benzene concentration after correction of dilution over initial concentration.

It has been reported that there are fewer microorganisms in groundwater than soil particles (Harvey et al. 1984). The groundwater was possibly not enriched with benzene-degrading microorganisms. The absence of benzene-degrading microorganisms may affect the lack of benzene biodegradation, and thereby resulting in a low benzene removal efficiency (Weiner and Lovley 1998). Most benzene-degrading lab microcosms were established from soil or sediments from contaminated sites (Vogt et al. 2011). Microcosms from soil or sediments usually show higher degradation rates and shorter lag time. Therefore, the source for inoculation may be important for benzene degradation to occur.

Benzene initial concentration may be another factor that resulted in low benzene degradation rate. Benzene was introduced at an initial concentration of around 25 mg/L. The effect of benzene initial concentration on degradation of benzene has been studied before (Edwards and Grbic-Galic 1992). Anaerobic benzene degradation microcosms were set up at initial concentration ranging from 40 to 200 μM. It was found that benzene degradation rate increased as initial concentration rose up to 140 μM. However at concentration of 200 μM, a longer lag time and much slower degradation rate were observed. The result suggested that when benzene concentration exceeds a certain level, it starts to show substrate toxicity impact on bacterial growth. Biodegradation was found inhibited by higher BTEX concentrations in a two thermophilic aerobic bacteria experiments (Chen and Taylor 1995). Duldhardt et al. (2007) reported a decrease in the growth rate and degradation kinetics of anaerobic bacteria due to exposure to high concentrations of organic hydrocarbons such as benzene and toluene.

Therefore, for further attempts, modifications of Recipe NP includes establishing microcosms inoculated with contaminated soil instead of groundwater, reducing initial benzene concentration from 25 mg/L to 17 mg/L, and stimulating anaerobic benzene degradation using sulfate instead of nitrate. Despite differences in methodology and substrate concentration, anaerobic benzene degradation under sulfate-reducing condition was observed in soil and sediments from aquifers, freshwater, or marine (Lovley et al. 1995; Coates et al. 1996; Kazumi et al. 1997; Phelps et al. 1996, 1998; Anderson and Lovely 2000; Morasch et al. 2001; Vogt et al. 2007; Kleinsteuber et al. 2008; Musat and Widdel 2008; Oka et al. 2008; Abu Laban et al. 2009; Berlendis et al. 2010; Herrmann et al. 2010).

Table 4.3 Benzene degradation in microcosms using Recipe NP inoculated with groundwater over 50 days. Dilution factor of benzene consumption subtracted for corrected final concentration.

	Benzene Concentration			
	Initial, mg/L	**Final, mg/L**	**Corrected Final, mg/L**	**Reduced Value, mg/L**
#NP1	27.42	22. 29	23.80	3.62
#NP2	25.40	21.31	22.6	2.80
Abiotic Control	25.19	21.18	22.54	2.65
Sterile Control	21.38	18.25	19.49	1.89

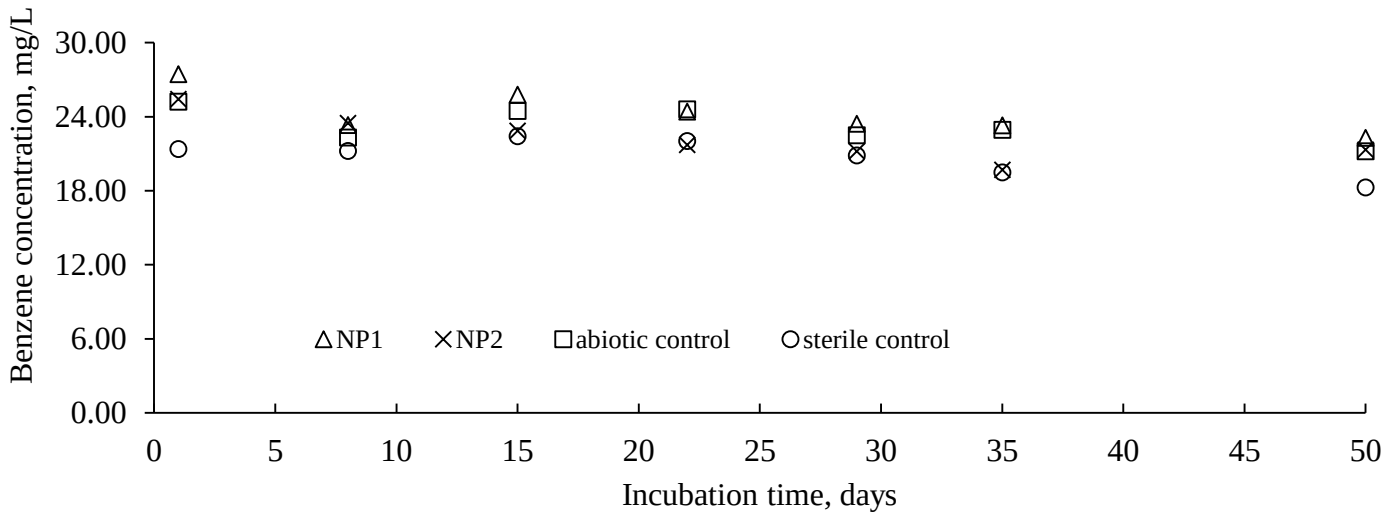

Figure 4.1 Benzene degradation in microcosms using Recipe NP inoculated with groundwater over 50 days.

4.2.2. Recipe Coates and Recipe Kazumi

According to the modifications based on previous experiment groups, Recipe Coates and Recipe Kazumi were used coupled with contaminated soil. Benzene degradation was not observed in microcosms either with Recipe Coates (Table 4.4; Figure 4.2) or Recipe Kazumi (Table 4.5; Figure 4.3) over 30 days. #C1 and #C2 represented two duplicate experimental group results using Recipe Coates. #K1 and #K2 represented two duplicate experimental group results using Recipe Kazumi.

Several reasons were speculated to affect benzene degradation performance. The first one was salinity. Since Recipe Coates and Recipe Kazumi were both to approximate the salinity of sea water (with addition of 20 g NaCl and 23 g NaCl, respectively), the presence of high salinity could be toxic to the microorganisms that play important roles in mineralization of benzene inoculated with soil collected from aquifer. The mineralization of benzene could be significantly inhibited by salinity. Painchaud et al. (1995) found that the growth of the freshwater bacteria was reduced by 15 % and 50 % after exposure to salinities of 10 and 20 ppm, respectively. The second factor was alternate carbon source. The inclusion of sodium acetate in Recipe Coates provided an alternate carbon source other than benzene that would likely confound the result. Also incubation time of 30 days might be too short for anaerobic benzene degradation to actually take place. Lag time for anaerobic benzene degradation was believed to be long and unpredictable (Edwards et al. 1992; Nales et al. 1998).

Modifications of Recipe Coates and Recipe Kazumi included removal of NaCl and sodium acetate. Contaminated soil was increased from 10 g to 100 g to form soil slurries (vol soil:vol media=10:90). In addition, three putative intermediates (toluene, phenol, and benzoate) were added to the cultures to test their effect on benzene degradation performance.

Table 4.4 Benzene degradation in microcosms using Recipe Coates inoculated with soil over 30 days. Dilution factor of benzene consumption subtracted.

	Benzene Concentration			
	Initial, mg/L	**Final, mg/L**	**Corrected Final, mg/L**	**Reduced Value, mg/L**
#C1	18.68	16.46	17.61	1.07
#C2	16.91	14.71	15.59	1.32
Abiotic Control	20.51	19.86	21.05	-0.54
Sterile Control	20.23	20.08	21.28	-1.05

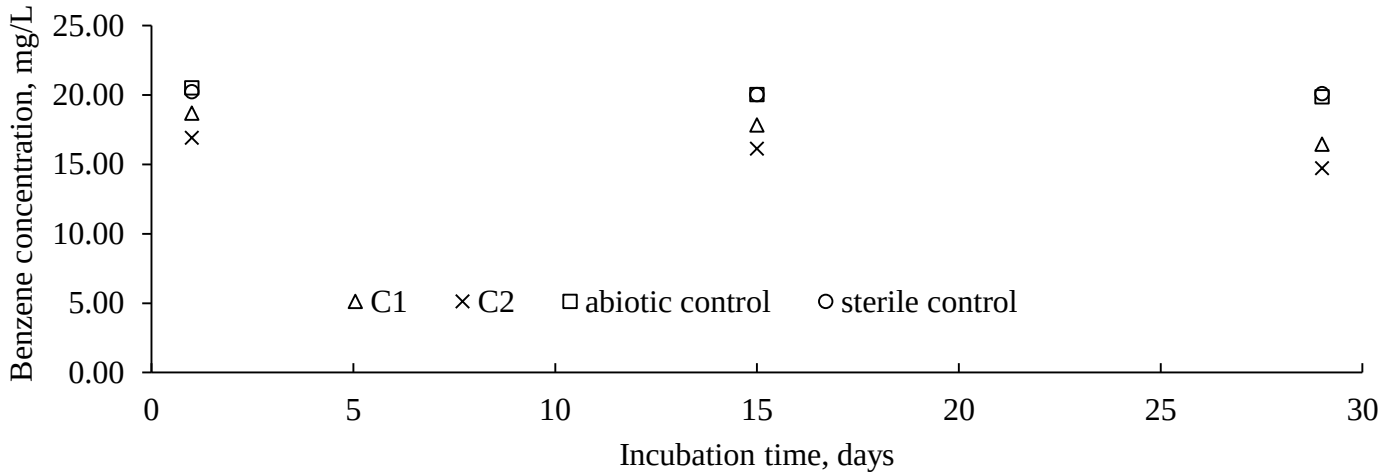

Figure 4.2 Benzene degradation in microcosms using Recipe Coates inoculated with soil over 30 days.

Table 4.5 Benzene degradation in microcosms using Recipe Kazumi inoculated with soil over 30 days. Dilution factor of benzene consumption subtracted.

	Benzene Concentration			
	Initial, mg/L	**Final, mg/L**	**Corrected Final, mg/L**	**Reduced Value, mg/L**
#K1	25.12	24.46	25.93	-0.81
#K2	26.93	26.71	28.31	-1.83
Abiotic Control	26.52	22.86	24.23	2.29
Sterile Control	25.66	25.08	26.58	-0.92

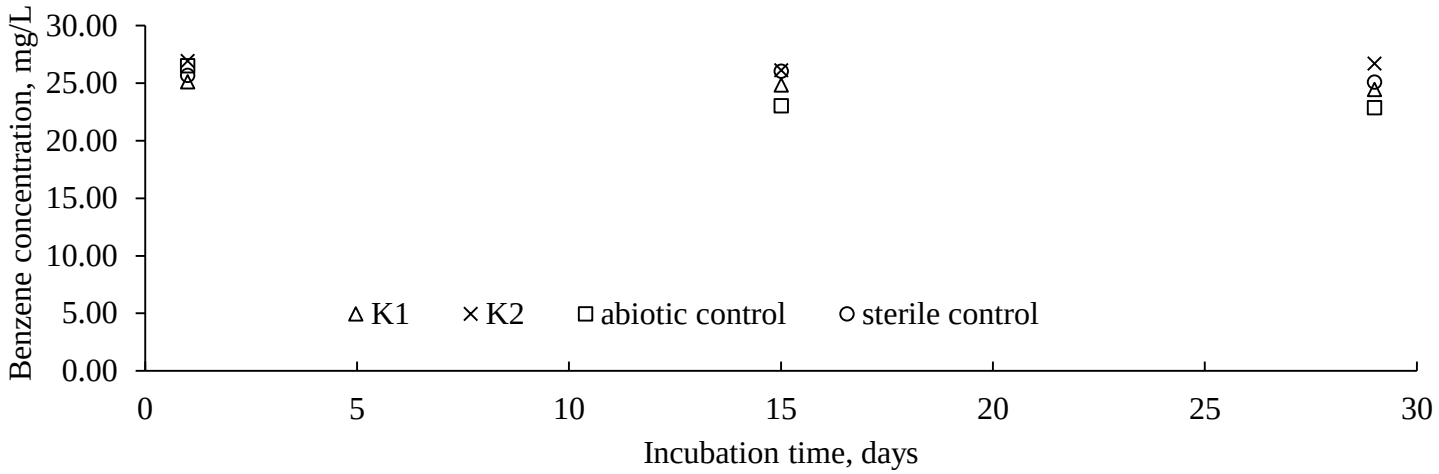

Figure 4.3 Benzene degradation in microcosms using Recipe Kazumi inoculated with soil over 30 days.

4.2.3. Recipe SA, Recipe SA+T, and Recipe SA+ P

According to modifications based on pervious experiments, benzene degradation was observed in microcosms inoculated with Recipe SA (Table 4.6; Figure 4.4), Recipe SA+T (Table 4.7; Figure 4.5), and Recipe SA +P (Table 4.8; Figure 4.6) during incubation time over 200 days. #SA1 and #SA2 represented two duplicate experimental groups using Recipe SA. #SAT1 and #SAT2 represented two duplicate experimental groups using Recipe SA+T. #SAP1 and #SAP2 represented two duplicate experimental groups using Recipe SA+P. Percentage of reduction was calculated in the same manner as described in Section 4.2.1.

In the first 35 days of incubation with Recipe SA, Recipe SA+T, and Recipe SA+P, benzene loss was hardly observed. The lag time was overcome by high concentration of nutrients amendment to a final concentration of 18 g/L K_2SO_4, 3.5 g/L KNO_3, and 28 g/L KH_2PO_4 within all three experimental cultures (as indicated by the arrows in Figure 4.4, Figure 4.5, and Figure 4.6). The nutrients amendment recipe was adapted from Xiong et al. (2012) where anaerobic benzene degradation was enhanced in field. After nutrients amendment, benzene degradation rate in all incubation cultures were found to be stimulated by an appreciable degree. This may be explained by that certain amounts of time and considerable quantities of limiting nutrients (nitrate, phosphate, and sulfate) were required for an initially small size of population to grow sufficiently large to achieve detectable benzene loss.

Figure 4.4, Figure 4.5, and Figure 4.6 show that benzene was degraded under all conditions with Recipe SA, Recipe SA+T, and Recipe SA+P, suggesting that anaerobic benzene degradation could occur with or without toluene, phenol, or benzoate amendments. Percentage of reduction over initial concentration for Recipe SA, Recipe SA+T, and Recipe SA+P was 18.08 %, 42.23 %, and 24.83 %, respectively (average of duplicates). Incubation with Recipe SA tends to have the lowest decrease value compared with the other two. Amendment with presumable anaerobic benzene degradation intermediates (i.e. benzoate, phenol, and toluene) seemed to stimulate anaerobic benzene degradation in this study. Recipe SA+T with toluene amendment achieved highest benzene degradation rate indicating toluene contributed to faster contaminant removal.

In order to gain more evidence of anaerobic benzene degradation and to identify dominant microorganisms responsible for anaerobic benzene degradation, BioTrap coupled with microbial analysis was employed. One duplicate with Recipe SA + T reached degradation percentage of

38.91 % which was the highest among six inoculations at that time. The inoculation with the highest reduction percentage was sacrificed for further analysis. It was mixed well before half volume was transformed to a new sterile experiment bottle. Then the bottle was filled to the top with anoxic media solution for BioTrap experiment. The preparation for incubation was described in detail in section 3.4.

Table 4.6 Benzene degradation in microcosms using Recipe SA inoculated with soil over 200 days. Dilution factor of benzene consumption subtracted.

	Benzene Concentration				
	Initial, mg/L	**Final, mg/L**	**Corrected Final, mg/L**	**Reduced Value, mg/L**	**%, Percentage of Reduction**
#SA1	16.93	10.13	13.62	3.31	19.55
#SA2	16.99	10.88	14.71	2.28	16.60

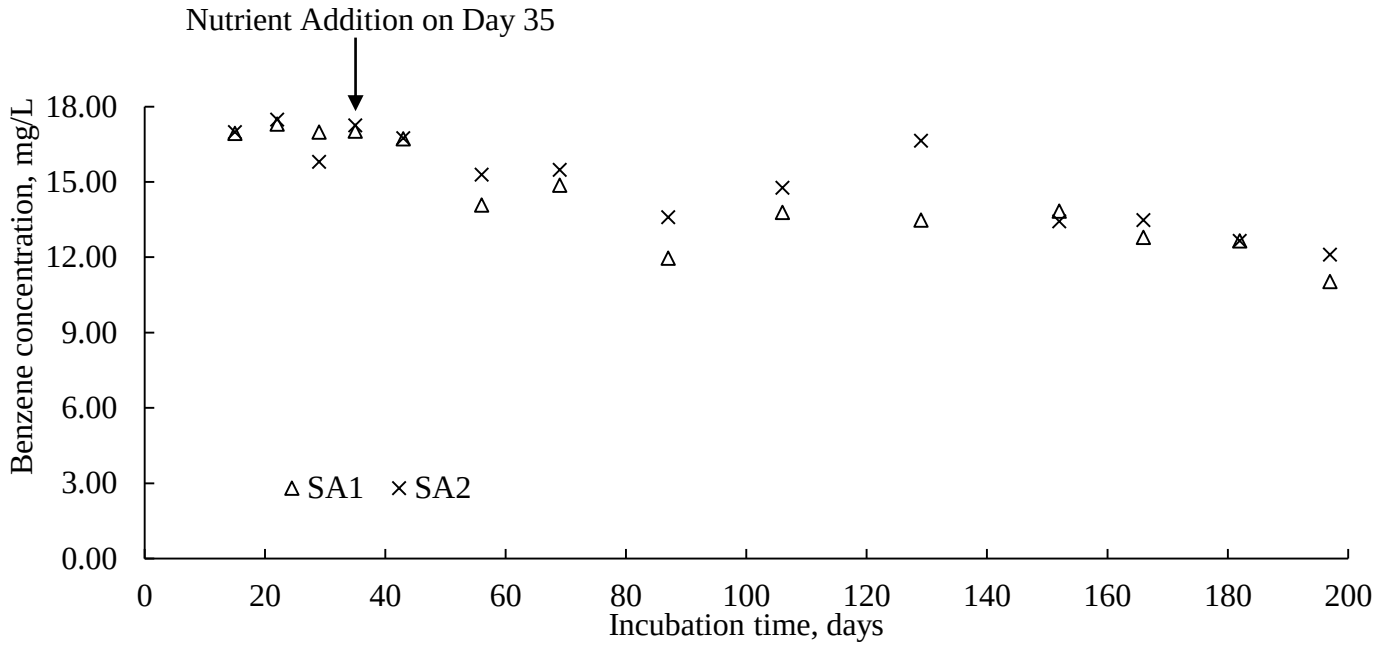

Figure 4.4 Benzene degradation in microcosms using Recipe SA inoculated with soil over 200 days. Arrow indicated day 35 with nutrients addition.

Table 4.7 Benzene degradation in microcosms using Recipe SA+T inoculated with soil over 200 days. Dilution factor of benzene consumption subtracted.

	Benzene Concentration				
	Initial, mg/L	**Final, mg/L**	**Corrected Final, mg/L**	**Reduced Value, mg/L**	**%, Percentage of Reduction**
#SAT1	15.01	8.01(day 166)	9.17	5.84	38.91
#SAT2	16.36	6.92	8.91	7.45	45.54

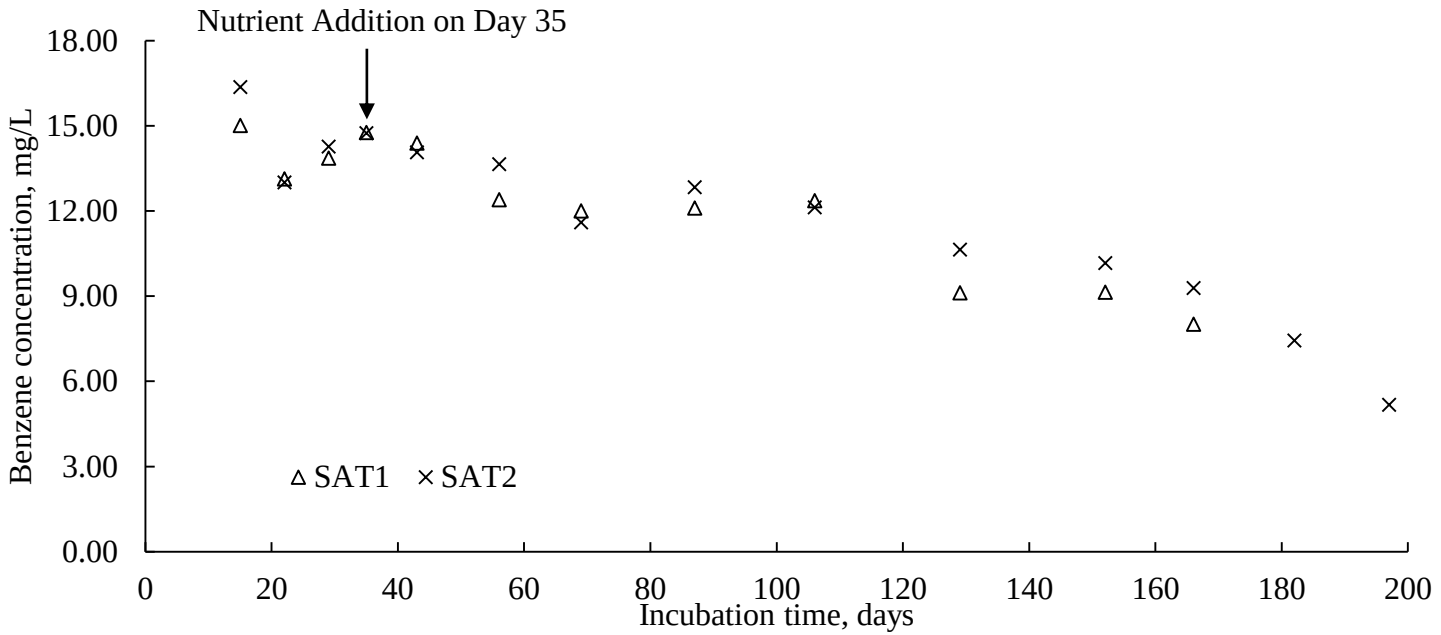

Figure 4.5 Benzene degradation in microcosms using Recipe SA+T inoculated with soil over 200 days. Arrow indicated day 35 with nutrients addition. One duplicate was sacrificed for BioTrap experiment on day 166.

Table 4.8 Benzene degradation in microcosms using Recipe SA+P inoculated with soil over 200 days. Dilution factor of benzene consumption subtracted.

	Benzene Concentration				
	Initial, mg/L	**Final, mg/L**	**Corrected Final, mg/L**	**Reduced Value, mg/L**	**%, Percentage of Reduction**
#SAP1	16.57	7.18	11.57	5.00	30.16
#SAP2	16.57	9.80	13.34	3.23	19.50

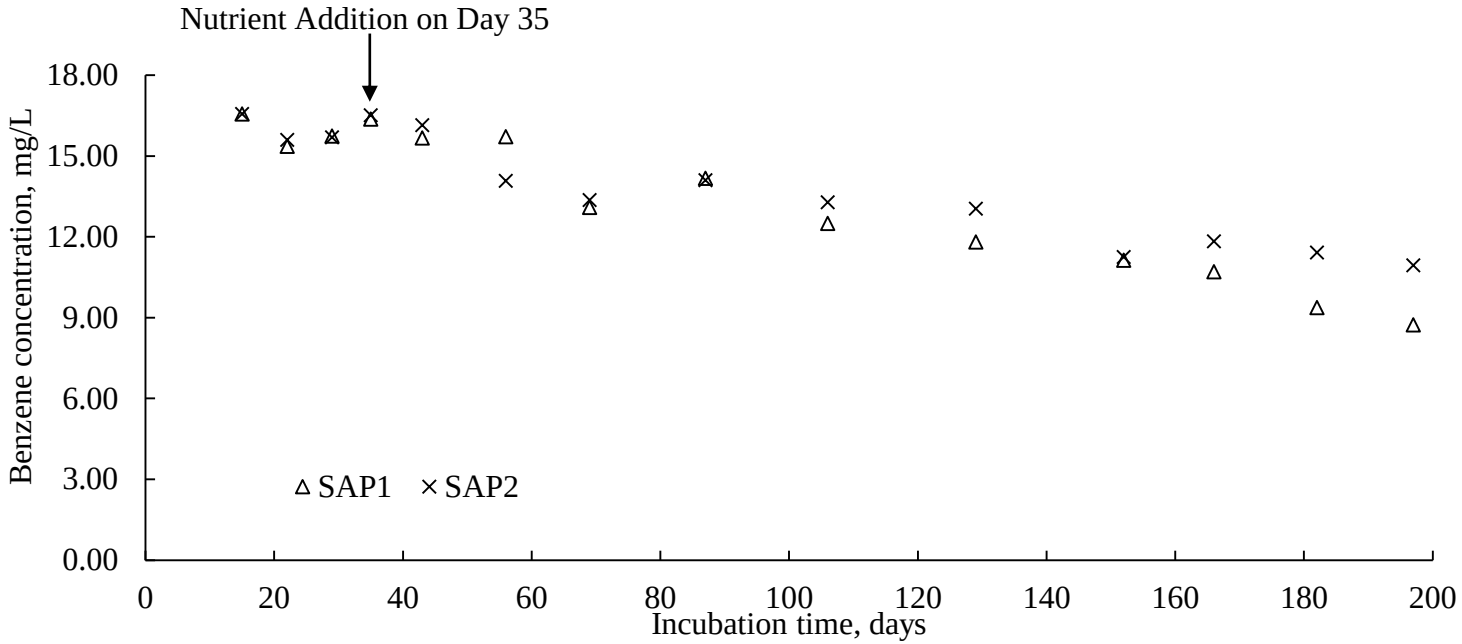

Figure 4.6 Benzene degradation in microcosms using Recipe SA+P inoculated with soil over 200 days. Arrow indicated day 35 with nutrients addition.

4.2.4. Summary

In this study, six recipes either under nitrate- or sulphate-reducing condition were tested. Indigenous microbes growing with Recipe SA, Recipe SA+T, and Recipe SA+P were able to reach effective anaerobic degradation of benzene (Table 4.9).

Table 4.9 Benzene degradation in microcosms with different recipes. + denotes benzene was degraded; − denotes no benzene degradation

Recipe	Effective Benzene Degradation
NP	−
Coates	−
Kazumi	−
SA	+
SA +T	+
SA +P	+

Cultures with Recipe SA+T were the most effective and efficient among all cultures. The composition of Recipe SA+T was per liter media 10 mg toluene, 18 g K_2SO_4, 3.5 g KNO_3, 28 g KH_2PO_4, 0.5 g NH_4Cl, 0.14 g $CaCl_2.2H_2O$, 1 g $MgCl_2.6H_2O$, 2.5 g $NaHCO_3$, 2 mg $FeCl_2.4H_2O$, 15 mL trace metal solution, and 10 mL vitamin stock. Recipe SA+T was considered to be the optimal medium composition for occurrence of anaerobic benzene degradation incubated with the contaminated soil collected from project site.

Since abiotic processes such as dilution, adsorption, and volatilization can contribute to hydrocarbon loss, criteria other than simple benzene loss should be used to evaluate overall benzene biodegradation performance. For example, increase in the number of benzene degraders as biodegradation processes can provide evidence of biodegradation. In this study, PLFA coupled with SIP, DGGE and sequencing, and qPCR were employed to uncover more evidence of anaerobic benzene degradation.

4.3. Contaminants Degradation in Culture with BioTrap

BioTrap combined with stable isotope probing has been proved to be an ideal method to determine whether biodegradation is occurring (Geyer et al. 2005; Busch-Harris et al. 2008; Fiorenza et al. 2009).

4.3.1. [^{12}C] Benzene and [^{12}C] Toluene Decrease

Both [^{12}C] benzene and [^{12}C] toluene reduction were observed in culture (Table 4.10; Figure 4.7). The initial concentration of [^{12}C] benzene and [^{12}C] toluene were around 17 mg/L and 5 mg/L, respectively. Benzene and toluene concentration were measured by GC/FID after 1, 15, 30, 40, 50, and 60 days. As can be seen from Fig. 4.7, toluene concentration dropped dramatically from 4.66 mg/L on day 0 to 0.23 mg/L on day 30. The concentration declined further down to 0.08 mg/L measured on day 60. Reduction percentage of 95.5 % of its initial concentration was observed during 60 days of incubation.

Similar pattern was found with benzene, which was observed to decrease from 16.92 mg/L on day 0 to 10.92 mg/L on day 30. Negligible concentration fluctuation was observed from day 30 to day 60. Final concentration was measured as 10.74 mg/L. Reduction percentage of 30.62 % of its initial concentration was observed.

Interestingly, both toluene and benzene were degraded effectively in a rapid manner during the first 30 days. After 30 days, benzene and toluene almost simultaneously stopped degradation activity. A correlation seems to exist between toluene degradation and benzene degradation.

Since toluene was reduced to a fairly low concentration that may not be sufficient to support further effective degradation, it is speculated the relatively stable benzene concentration was caused by depleted toluene concentration. Toluene was assumed to be an essential co-substance for anaerobic benzene degradation. Similar pattern has been seen with other aromatic compounds. Generally, high molecular weight PAHs are only biodegraded in the presence of other low molecular weight hydrocarbons or complex hydrocarbon mixtures such as crude oil. If the necessary co-substrates are absent, the co-metabolic biodegradation of higher molecular weight PAHs cannot proceed (Singh et al. 2005).

Since both [^{12}C] benzene and [^{12}C] toluene were degraded by 30.6 % and 95 % on day 30 respectively and preserved steady for the next 30 days, no further decline of benzene or toluene

was expected. The BioTrap was retrieved on day 60 and immediately sent to Microbial Insight overnight for further stable isotope probing related analysis and molecular biological analysis.

Table 4.10 Benzene and toluene degradation in microcosm using Recipe SA+P with BioTrap submerged for 60 days. Dilution factor of benzene consumption subtracted.

	Concentration				
	Initial, mg/L	**Final, mg/L**	**Corrected Final, mg/L**	**Reduced Value, mg/L**	**%, Percentage of Reduction**
Benzene	16.92	10.74	11.74	5.18	30.62
Toluene	4.66	0.08	0.21	4.45	95.50

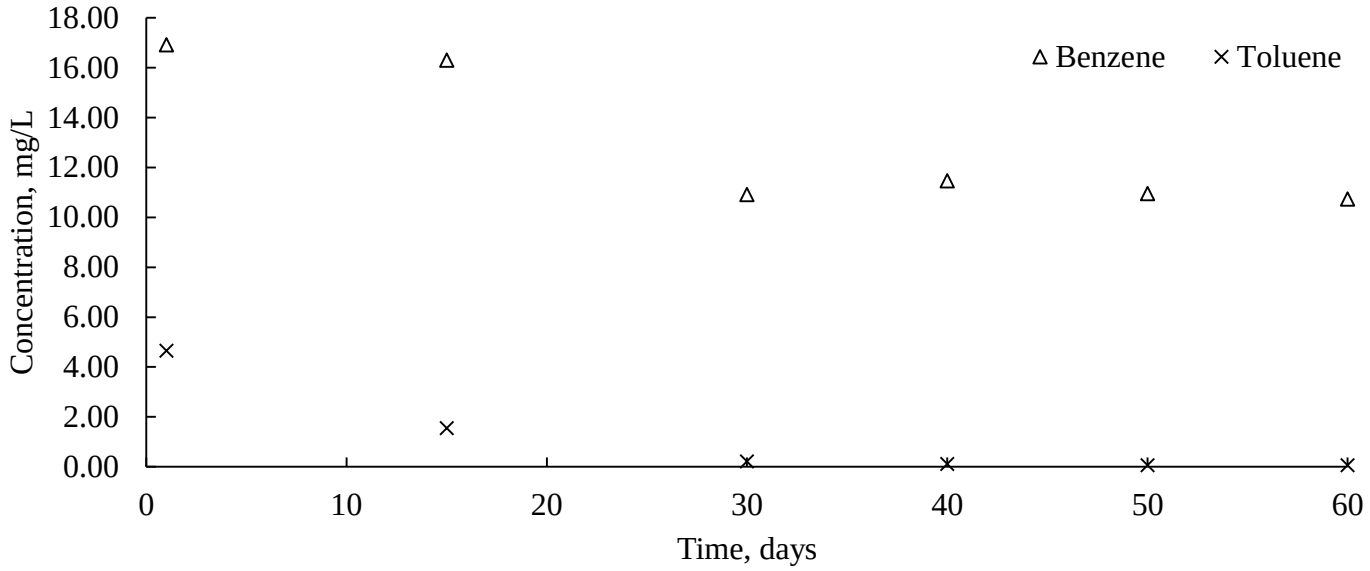

Figure 4.7 Benzene and toluene degradation in microcosm using Recipe SA+P with BioTrap submerged for 60 days

4.3.2. [^{13}C] Benzene Decrease

BioTrap BioSep beads were pre-baited with [^{13}C] benzene. The pre-deployment value was 192 µg/bead ±11 µg/bead (mean ± standard deviation). This value was determined in Microbial Insight before shipping. Following 60 days of incubation, the BioTrap was recovered, and [^{13}C] benzene remaining concentration was measured as 124 µg/bead ±11 µg/bead by Microbial Insight. The pre- and post-deployment concentration were used to calculate percent loss, which is 35.42 % (Figure 4.8). The loss of the [^{13}C] benzene provides an estimate of the degradation rate.

[^{12}C] benzene and [^{13}C] benzene in culture showed degradation percentage of 30.62 % and 35.42 % after 60 days of incubation, respectively. A slightly higher degradation rate with [^{13}C] benzene indicates BioTrap BioSep beads were beneficial for bacteria growth by providing large internal surface area to live in and easy access to necessary nutrients.

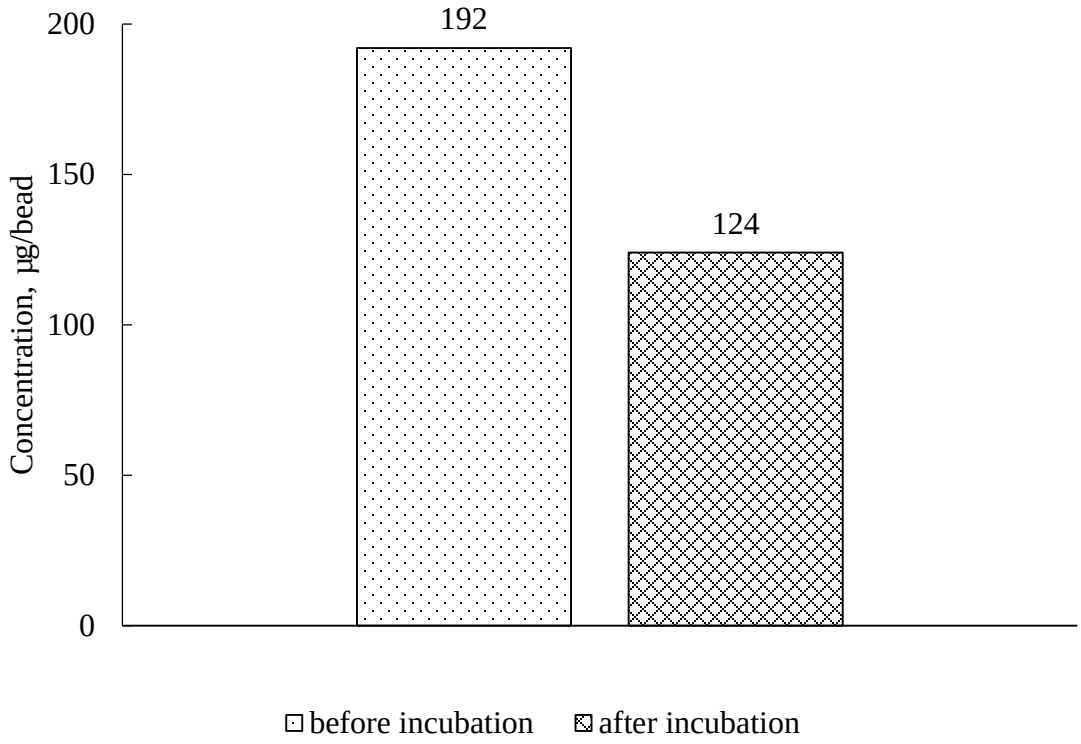

Figure 4.8 Comparison of pre- and post-deployment [^{13}C] concentration

4.3.3. Total Biomass and [^{13}C] Enriched Biomass in PLFA

BioTrap sampler is a passive sampler containing BioSep beads that collect microbes over time to better understand contaminant biodegradation potential. When combined with SIP, it can provide proof of benzene degradation within the microorganisms. PLFAs are biomarkers for microbial membrane phosphoglycerolipid content, which are indicators for viable (live) biomass (Guckert et al. 1985). Phospholipids break down rapidly after cell death, so biomass calculations based on PLFA content do not include dead cells. Total biomass (cells/bead) was calculated from total PLFA using a conversion factor of 20,000 cells/pmol of PLFA. The total biomass was measured to be 3.65E+06 cells/bead, indicating a significant microbial colonization in the BioTrap. [^{13}C] enriched biomass was measured to be 6.9E+04 cells/bead (Figure 4.9).

[^{13}C] enriched PLFA is often reported as δ^{13}C‰ (Hanson et al. 1999; Pelz et al. 2001; Johnsen et al. 2002; Geyer et al. 2005). Delta (δ) is the difference between the isotopic ratio (^{13}C/^{12}C) of the sample (R_x) and a standard (R_{std}) normalized to the isotopic ratio of the standard (R_{std}) and multiplied by 1000, which is expressed as

$$\delta^{13}C‰ = {(R_x - R_{std})}/{R_{std}} \times 1000 \quad (4.1)$$

The isotopic ratio, R_x, of PLFA is usually less than the R_{std} under natural conditions, resulting in a δ^{13}C value between -20 ‰ and -30 ‰ (Pelz et al. 2001; Geyer et al. 2005).

In this study, high level of [^{13}C] incorporation into microbial PLFA was showed in [^{13}C] benzene-amended BioTrap with an average value of 995 ‰ and a maximum value of 3192 ‰. This result directly demonstrates benzene biodegradation and quantifies [^{13}C] incorporation into biomass. Since the beads were sterile and free of biomass before deployment, an increase in [^{13}C] in the biomass strongly suggests that the bacteria incorporated the [^{13}C] from the [^{13}C] benzene into their cell mass, thus demonstrates biodegradation of [^{13}C] benzene. [^{13}C] incorporation into biomass is a result of [^{13}C] being used in cellular growth. The microorganisms that contain [^{13}C] label may be the primary degraders, or consume labelled intermediates produced from metabolic, co-metabolic, or abiotic transformation of the labelled compounds (Boschker and Middelburg, 2002).

The percentage of [^{13}C] incorporation, which is expressed by [^{13}C] enriched biomass/total biomass, was 1.89 %. A small number as it may seem, this value must be interpreted carefully since

typically biodegradation is performed by a small part of the total microbial community. For BioTrap with large total biomass, the percentage of [^{13}C] incorporation could be low despite significant [^{13}C] incorporation into biomass.

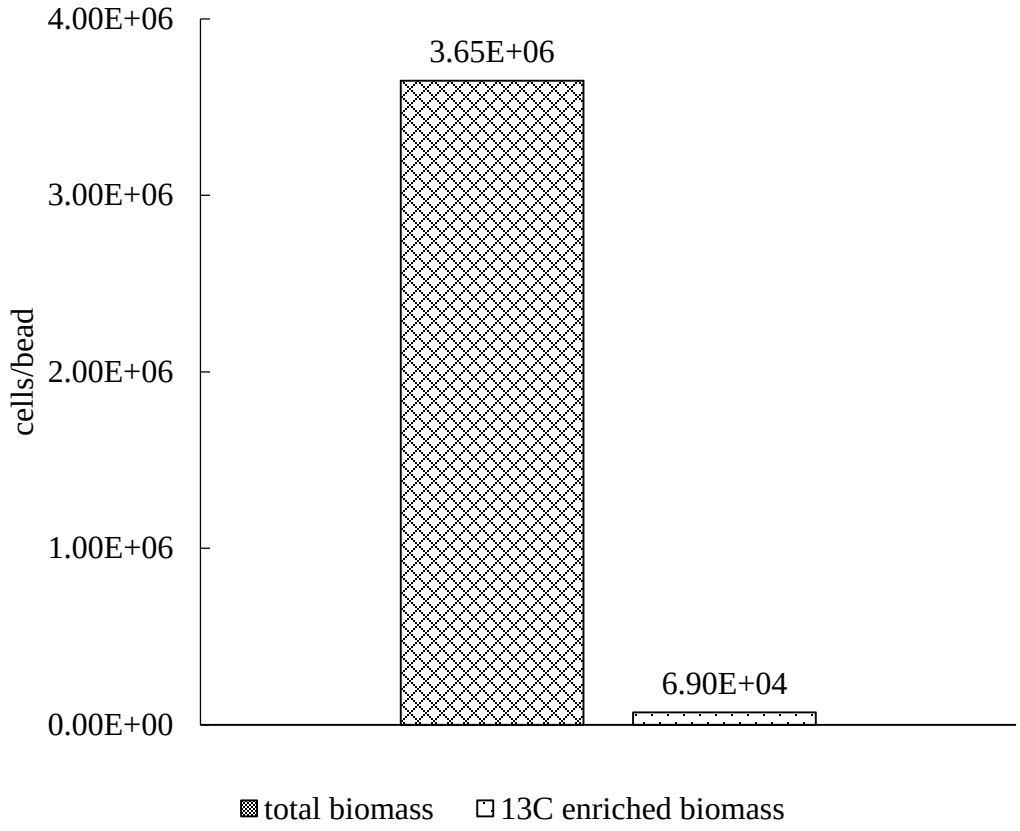

Figure 4.9 Total and [^{13}C] enriched biomass.

4.3.4. Dissolved Inorganic Carbon

Often, bacteria can utilize the organic compounds as carbon and energy source. The portion used as carbon source for cellular growth is incorporated into PLFA, while the portion used as energy source is oxidized to carbon dioxide as dissolved inorganic carbon (DIC). DIC is a measure of the amount of the organic carbon that was completely mineralized to its final products. Due to analytical procedure, DIC result represented gaseous CO_2 including H_2CO_3 evolved CO_2.

[^{13}C] enriched DIC is often reported as δ^{13}C as described before for PLFA. Under natural condition, the δ^{13}C value of CO_2 ranges between -26 ‰ and 0 ‰ (Shultz and Calder 1976; Fritz et al. 1978). In this study, a positive value of 20 ‰ in SIP DIC analysis was detected in the BioTrap after 60 days of incubation indicating that [^{13}C] benzene has been mineralized. The percentage of minerlalization is not considered significant suggesting that [^{13}C] benzene is only partially mineralized in the culture. More time may be required for completing [^{13}C] benzene mineralization.

4.3.5. Summary

For SIP study, ^{13}C incorporation can be viewed in light of [^{13}C] benzene percent loss, [^{13}C] enriched biomass vs. total biomass, and dissolved inorganic carbon results altogether. In this study, 30.62 % [^{12}C] benzene and 35.42 % [^{13}C] benzene loss were observed. Total biomass for culture community within BioTrap was measured as 3.65E+06 cells/bead indicating a significant population in the BioTrap. [^{13}C] enriched biomass was 6.90E+04 cells/bead. PLFA δ^{13} value of 995 ‰ shows a high level of benzene incorporation. DIC δ^{13} value of 20 ‰ fell into a lower range nevertheless shows benzene mineralization. These collective results clearly demonstrate that indigenous microorganisms from the contaminated soil were able to biodegrade and mineralize benzene. In order to identify the microorganisms responsible for benzene biodegradation and mineralization, the microbial community structure was characterized with PLFA, qPCR, DGGE, and Sequencing.

4.4. Culture Community Structure

Molecular fingerprinting techniques were used to study the ecology of the consortia and to identify the majority constitution of microorganisms.

4.4.1. PLFA

Community structure can be presented as a percentage of PLFA structural groups biomass normalized to total PLFA biomass (Pelz et al. 2001; Hedrick et al. 2005). Some organisms can produce signature PLFA allowing quantification of important microbial functional groups. The relative proportions of PLFA structural groups provide a fingerprint of the microbial community in culture, and therefore offer insight into the dominant metabolic processes. Community structure is presented as a percentage of PLFA structural groups normalized to the total PLFA biomass (Table 4.11).

Table 4.11 Microbial community structure groups outlined using % total PLFA

Community Structure Groups	% of Total
Firmucutes (TerBrSats)	2.8
Proteobacteria (Monos)	63.1
Anaerobic metal reducers (BrMonos)	1.1
Actinomycetes (MidBrSats)	0.2
General (Nsats)	32.9
Eukaryotes (Polyenoics)	0.0

The most abundant microbial group fell in Monos (63.1 % of total). Monos (Monoenoic fatty acids) are common to most bacteria, and abundant in Proteobacteria (gram negative bacteria). They typically grow fast, utilize many carbon sources, and adapt quickly to a variety of environments. High percentage of Monos within the bacterial community structure clearly indicates the presence of hydrocarbon-utilizing bacteria. The next abundant microbial group fell in Nsats (32.9 % of total). Nsats are found in all organisms, which often indicate diversity of microbial populations.

TerBrSats are common to Firmicutes (low G+C gram-positive bacteria) and Actinomycetes (high G+C gram-positive bacteria), and are also found in anaerobic Proteobacteria, some Proteobacterial facultative anaerobes, and some Gram-negative bacteria (Kaneda 1991). TerBrSats took up 2.8 % of total biomass, which can act as an indicative of anaerobic conditions. BrMonos (Branched Monoenoic) are found in cell membranes of microaerophiles and anaerobes, such as

sulfate- or iron-reducing bacteria. MidBrSats are commonly found in sulfate-reducing bacteria (SRB). The less abundance of BrMonos and MidBrSats indicates that biodegradation in the culture is not likely to be associated with anaerobic sulfate- or iron-reducing conditions. Polyenoic is found in higher plants and animals, which often prey on contaminant utilizing bacteria. Nearly no Polyenoics were found in the culture. It is expected since the small pore size of the membrane outside the BioSep beads generally omits eukaryotes.

In this study, the PLFA community structure was dominated by Monos, indicators of the diverse bacterial group *Proteobacteria* (63.1 %). A high percentage of normal saturate PLFA biomarkers were also detected (32.9 %). The PLFA results provide broad groups of microorganisms' identification. While this information does not directly identify microorganisms that are responsible for [^{13}C] benzene degradation in the BioTrap, the decrease of [^{13}C] benzene can still be linked to the increase in microbial biomass. In order to fully explore the microbial populations that might be involved in anaerobic benzene degradation, the microbial community was analyzed with qPCR and DGGE in more detail.

4.4.2. Quantitative Polymerase Chain Reaction

Quantitative polymer chain reaction (qPCR) is a rapid detection and quantification method for specific microorganisms, groups of microorganisms, or functional genes involved in bioremediation process. In this study, qPCR was performed on several genes targeting for specific degradation functions. The results are shown in Table 4.12.

Table 4.12 Microbial population outlined by functional genes and phylogenetic group

Functional Genes	**Gene Copies/Beads**
Benzene carboxylase (abcA)	<5.00E+01
Benzylsuccinate synthase (bssA)	<5.00E+01
nirS	2.01E+06
nirK	3.05E+07
Phylogenetic Group	**Gene Copies/Beads**
APS	5.13E+02

Benzylsuccinate synthase is the key enzyme of anaerobic toluene degradation, and has been found in all isolated anaerobic toluene degradation bacteria that have been tested (Winderl et al. 2007). The benzylsuccinate synthase alpha-subunit (bssA) gene encodes a Benzylsuccinate

synthase that was associated with the first step of anaerobic toluene degradation, thus allows for the specific detection and affiliation of both known and unknown anaerobic toluene degraders (Beller et al. 2002). The limited amount of bssA gene shows limited amount of anaerobic toluene degraders. It agrees with previous speculation that fast depletion of toluene in first 30 days resulted in inadequate toluene bioavailability and low toluene biodegradation activity when retrieved at day 60.

Biomarkers linked to anaerobic benzene degradation are however not conclusively clear yet. The first use of qPCR for forensic analysis of anaerobic benzene degradation was by Da Silva and Alvarez (2007). A 16S rRNA biomarker, *Desulfobacterium* sp. clone OR-M2, was designed from a methanogenic benzene-degrading consortium that has been enriched on benzene for several years (Da Silva and Alvarez 2007). Although *Desulfobacterium* sp. has been disproved as a benzene degrader, its enrichment as a result of benzene consumption and its correlation to anaerobic benzene degradation activity suggest that *Desulfobacterium* sp. either initiates or acts as a critical partner in benzene degradation. More recently, a specific benzene-expressed protein named anaerobic benzene carboxylase (abcA) was identified from a highly enriched iron-reducing benzene-degrading culture composed of mainly *Peptococcaceae*-related Gram-positive microorganisms (Abu Laban et al. 2010). Initial activation reaction to break benzene ring under anaerobic condition was proposed as a direct carboxylation catalyzed by benzene carboxylase, where its production is further activated by Benzoate-CoA ligase Bam Y to benzoate-CoA.

Since neither gene has been proved to be adequately selective or broadly applicable to assess anaerobic benzene degradation, abcA was chosen to be tested for the presence of anaerobic benzene degraders in this study. The result showed that insufficient abcA gene was found. Since abcA gene is not universal to target anaerobic benzene degrader, the result only suggests carboxylation may not be the initial reaction for benzene degradation in this study. The anaerobic benzene carboxylation inactivity on day 60 could be the result of fast depletion of toluene in the first 30 days and limited availability of toluene.

Reduction of nitrite to nitric oxide is catalyzed by two different nitrite reductase genes, nirK and nirS (Braker et al. 1998; Kandeler et al. 2006). Amplification of these two specific nitrite reductase genes fragments with PCR was used for the detection of denitrifying bacteria. A substantial amount of nirS and nirK were detected at 10^6 to 10^7 gene copies/bead, respectively. The

result supports population of denitrifying bacteria. Many denitrifiers anaerobically degrade hydrocarbons (Heider 2007). The only isolated anaerobic benzene degraders are denitrifying bacteria (Coates et al. 2001; Kasai et al. 2006).

For quantification of sulfate-reducing bacteria (SRB), adenosine-5′-phosphosulfate reductase (APS) is generally targeted (Barton and Fauque 2009; Wagner et al. 2005; Akinnibosun and Burton 2012). Anaerobic benzene degradation has been observed under sulfate-reducing condition (Berlendis et al. 2010; Herrmann et al. 2010; Kleinsteuber et al. 2008; Laban et al. 2009; Musat and Widdel 2008; Oka et al. 2008; Phelps et al. 1998). APS genes were detected as 5.13E+02 gene copies/bead in this study implying that SRB are also members of the microorganisms living within the culture. The low amount of APS corresponds to the finding of low Branched Monoenoic MidBrSats found in total biomass. This is unexpected considering high concentration of sulfate in the culture. Whether sulfate served as inorganic nutrient rather than electron acceptor to support overall bacterial growth remained to be ruled out. Some researchers have reported the inhibiting effect of denitrifying bacteria on sulfate-reducing process (Yan et al. 2007; Li et al. 2012; Zhang et al. 2013).

4.4.3. DGGE and Sequencing

To determine bacterial community patterns in BioTrap, DGGE and sequencing technique were performed. Although comparative analysis of 16S rRNA sequences does not allow definitive determination of which microorganisms are responsible for a certain biogeochemical process, this approach is clearly effective in identifying the major genus in the microbial community (Teske et al. 1996). Figure 4.10 is a DGGE gel image showing bacterial community profiles of BioTrap after 60-days' incubation. Weaker bands were difficult to be cut off from the gel and therefore hard to be re-amplified. Successfully sequenced 16S rRNA genes were compared to the database of GenBank and Ribosomal Database Project (RDP), whose results were given in Table 4.13. An analyzed sequence with similarity indices above .900 is considered excellent, .700−.800 is good, and below .600 are considered a potentially unique sequence.

Table 4.13 Band identification for major DGGE bands appearing on Figure 4.10.

Band	Similar Genus	Similarity Index	Affiliation	GenBank Accession Number
1.1	*Pusillimonas spp.*	0.926	*Betaproteobacteria; Alcaligenaceae*	FN667020.1
1.2	*Advenella spp.*	0.880	*Betaproteobacteria; Alcaligenaceae*	KC464861.1; KC207092.1; JQ799008.1
1.3	*Pusillimonas spp.*	0.990	*Betaproteobacteria; Alcaligenaceae*	KC464818.1; HQ326782.1; FN667020.1
1.4	*Pusillimonas spp.*	0.923	*Betaproteobacteria; Alcaligenaceae*	FM956659; FJ791048; GQ246953
1.5	*Dokdonella spp.*	0.980	*Gammaproteobacteria; Xanthomonadaceae*	JQ726695.1; JQ726692.1; JQ726691.1
1.6	*Dokdonella spp.*	0.970	*Gammaproteobacteria; Xanthomonadaceae*	JQ726695.1; JQ726692.1; JQ726691.1
1.7	*Dokdonella spp.*	0.876	*Gammaproteobacteria; Xanthomonadaceae*	AY921834.1

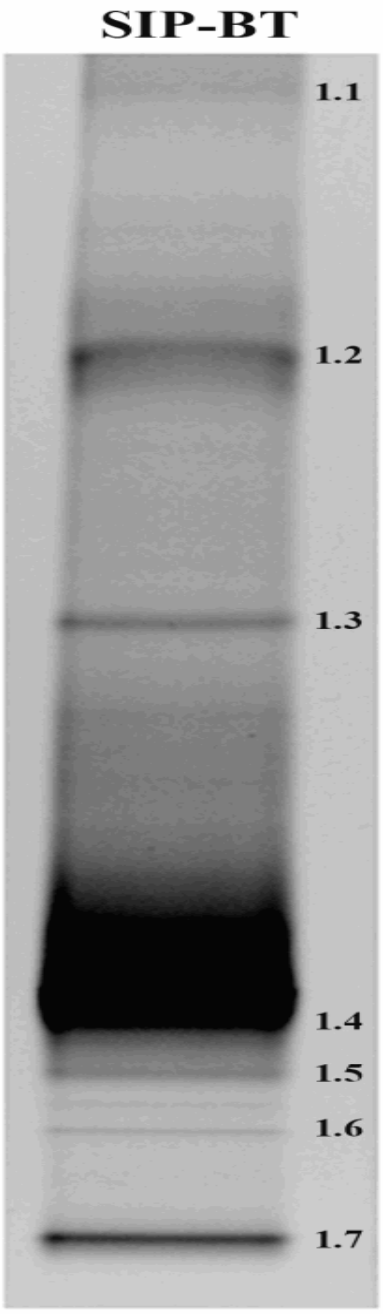

Figure 4.10 DGGE gel image of microorganisms in BioTrap. Refer to Table 4.13 for band identification.

The DGGE analysis of 16S rDNA retrieved revealed that the most distinct bands could be assigned to three major groups, *Dokdonella spp.*, *Pusillimonas spp.*, and *Advenella spp.*, whose phylogeny affiliate to *Betaproteobacteria* and *Gammaproteobacteria*. Members of family *Betaproteobacteria* and *Gammaproteobacteria* are known to utilize aromatic compounds. Phylogenetic group from previous pure-culture studies under nitrate-reducing condition also affiliated to *Betaproteobacteria* (Coates et al. 2001; Kasai et al. 2006). Ulrich and Edwards (2003) found dominant phylotypes in benzene-degrading cultures were *Betaproteobacteria*. Several sequences from the culture shared close homologies with environmental samples detected in phenol-degrading microcosm from contaminated aquifer, hydrocarbon contaminated soil, water treatment facilities, and oil and gas field.

Band 1.1, band 1.3, and band 1.4 had similarity index of 0.926, 0.990, and 0.923 respectively with *Pusillimonas spp.*, whose DNA falls within the order *Proteobacteria*, and particularly within the family of *Alcaligenaceae*. Some identified members of genus *Pusillimonas* are anaerobic bacteria that grow on either phenol or other hydrocarbons (Elliott et al. 2010; Zhang et al. 2012). *Pusillimonas* sp. T7-7 is a diesel-degrading cold-tolerant bacterium isolated from the benthal mud of a PHC-contaminated site (Cao et al. 2011). A DGGE band affiliated with *Pusillimonas spp.* was found in an anaerobic toluene-degrading culture (Sun et al. 2013). *Pusillimonas noertemannii* is an aromatic compounds degrader such as benzoic acid, which is a prominent intermediate metabolite of many aromatic hydrocarbon biodegradation (Stolz et al. 2005; Hilyard et al. 2008).

Band 1.2 had a 0.88 identity to *Advenella spp.*. The closest isolated bacteria to *Advenella spp.* in the16S rRNA gene clone library falls within the order *Betaproteobacteria*, and particularly within the family of *Alcaligenaceae*.

Band 1.5, band 1.6, and band 1.7 had similarity index of 0.98, 0.97, and 0.876 respectively to *Dokdonella spp.*. The closest isolated bacteria to *Dokdonella spp.* in the 16S rRNA gene clone library falls within the order *Gammaproteobacteria*, and particularly within the family of *Xanthomonadaceae*. Genus *Dokdonella* is commonly isolated from soil or activated sludge, and has the ability to reduce nitrate under anaerobic condition (Ten et al. 2009; Yoon et al. 2006). Many members from *Dokdonella spp.* are denitrifying bacteria. *Dokdonella spp.* was found in environmental samples collected from harbour sediments growing on TNT under anaerobic condition (Gallagher et al. 2010).

4.5. General Discussion and Synthesis

Like many other aromatic hydrocarbons, benzene is a common environmental contaminant and its exposure negatively impacts human health. Among existing treatment methods for contaminated sites, in situ bioremediation that converts organic pollutants to non-toxic substances by microorganisms is a cost-effective and efficient method. Growth of key microorganisms that are responsible for degradation of contaminant of interest is essential for efficient bioremediation of contaminated sites. Identification of the key microorganisms is of great importance to understanding anaerobic degradation of benzene. Thus the objective of current study was to identify dominant microorganisms involved in anaerobic benzene degradation.

4.5.1. Establishing Optimum Growth of Benzene-Degrading Cultures

Benzene-degrading cultures were established from contaminated soil and groundwater that were collected from the selected project site. Several media recipes were tested to investigate the optimum growth condition for indigenous microorganisms which were responsible for anaerobic benzene degradation.

Several factors were assumed to negatively impact anaerobic benzene degradation performance. One factor was the source for inoculation. Cultures established with soil and sediment usually have better degradation performance than those with groundwater. It was found that there are fewer microorganisms in groundwater than in soil particles (Harvey et al. 1984). Another factor was the culturing time. The lag time for the occurrence of anaerobic benzene degradation is usually long and unpredictable (Edwards and Grbic-Galic 1992; Vogt et al. 2011). It varies from case to case and site to site. Initial concentration of benzene may be another factor that influenced benzene degradation rate. Biodegradation process was found inhibited by higher concentration of BTEX (Chen and Taylor 1995). The presence of salinity could also inhibit the growth of microorganisms originated from soil and groundwater (Painchaud et al. 1995).

With modifications from previous experiment trails, cultures with Recipe SA+T was considered to be the most effective and efficient medium composition for the occurrence of anaerobic benzene degradation incubated with the contaminated soil from the project site. Large dosage of nutrients would potentially shorten the lag time and simulate benzene degradation. When benzene was depleted to half of its initial concentration, one duplicate culture was sacrificed for microbial chemical analyses to investigate the anaerobic benzene-degrading culture.

4.5.2. Characterizing Biostimulated Benzene-Degrading Cultures

Anaerobic benzene-degrading cultures derived from contaminated site represent complex microbial communities. Characterizing the biostimulated benzene-degrading cultures and in particular identifying dominant members that are responsible for benzene degradation can lead to development of cultures with high degradation capacity that can potentially be applied to contaminated sites.

A BioTrap coupled with SIP study was performed to determine whether benzene biodegradation was occurring in culture after 60 days' incubation. As discussed in Section 4.3 and Section 4.4, quantification of contaminant loss ([^{13}C] benzene), [^{13}C] enriched biomass and total biomass, and DIC clearly demonstrated that benzene biodegradation mineralization occurred.

The DGGE analysis of 16S rDNA retrieved from the culture revealed that the most distinct bands in the microbial community were assigned to three major groups, *Dokdonella spp.*, *Pusillimonas spp.*, and *Advenella spp.*, whose phylogeny affiliate to *Betaproteobacteria* and *Gammaproteobacteria*. Studies showed most members from these three groups were denitrifying bacteria. Phylogenetic group from previous pure-culture studies under nitrate-reducing condition also affiliated to *Betaproteobacteria* (Coates et al. 2001; Kasai et al. 2006). The same results were also shown with qPCR analysis. A substantial amount of denitrifying bacteria (nirS and nirK) was detected. PLFA results showed the majority of the microbial community were hydrocarbon-utilizing bacteria. The results of qPCR, PLFA, and DGGE collectively indicated that members of *Betaproteobacteria* are very likely to be primary benzene degraders or supported benzene degraders' growth.

Chapter 5. CONCLUSIONS AND FUTURE WORK

5.1. Conclusions

In conclusion, this study successfully identified dominant indigenous microorganisms that can degrade benzene under anaerobic condition. The first objective was to find the optimum condition to stimulate benzene degradation. The results suggest that salinity and high initial concentration of benzene could affect anaerobic benzene degradation activity. On the other hand, benzene degradation performance could be enhanced by addition of limiting nutrients. Large dose of nutrient amendment could stimulate the growth of benzene-degrading bacteria and in the meanwhile shorten the lag time for actual occurrence of benzene degradation. A modified medium recipe was formulated to sustain anaerobic benzene degradation. Benzene-degrading cultures were successfully established and maintained over 200 days with a continuous decreasing trend. The results demonstrate that the potential for anaerobic benzene biodegradation existed indigenously. Putative intermediates (i.e. toluene, phenol and benzoate) were found to promote benzene degradation activity. Higher overall benzene removal percentage was found in culture with than without putative intermediates amended. Of three putative intermediates, cultures with toluene showed the highest removal percentage. The results strongly suggest that toluene was an essential co-substance for anaerobic benzene degradation in this study.

The second objective was to identify dominant members presented in the microbial community during benzene biodegradation. BioSep BioTrap coupled with stable isotope probing was applied. The SIP study provided direct evidence of benzene biodegradation and mineralization in the BioTrap. Comparison of pre- and post-deployment [^{13}C] benzene in BioSep beads showed about a 35 % loss. The average DIC δ^{13}C (20 ‰)

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demonstrate benzene mineralization. These collective results demonstrate that the potential for anaerobic benzene biodegradation existed indigenously.

The PLFA profiles of viable total biomass confirmed that the microbial community structure was dominated by monounsaturates, indicators of the diverse bacterial group *Proteobacteria*. The dominant indigenous microorganisms were identified by PCR-DGGE analysis. DGGE results showed that members of genus *Dokdonella spp.*, *Pusillimonas spp.*, and *Advenella spp.*, whose phylogeny affiliated to *Betaproteobacteria* and *Gammaproteobacteria* were dominant in microbial community. It is very likely that these microorganisms either were primary benzene degraders or supported benzene degraders' growth. The supporting evidences include (i) the significant amount of *Betaproteobacteria* and *Gammaproteobacteria* within the BioTrap BioSep beads, (ii) *Betaproteobacteria* contains the only known isolated anaerobic benzene degrader, (iii) the finding that *Betaproteobacteria* species were dominant in a benzene-degrading nitrate-reducing culture established with benzene contaminated soil. Quantitative PCR results showed a substantial amount of denitrifying bacteria within the culture. SRB were also active members in the community. The discovered microbial population of denitrifying bacteria and SRB implied that collected soil samples contained a variety of microorganisms which used different mechanisms to degrade benzene or toluene or their metabolites.

In summary, this study successfully established a nitrite-reducing benzene-degrading culture with relatively high degradation potential. The research conducted in this thesis can yield insights into anaerobic benzene biodegradation in terms of identification of dominant microorganisms involved in the process, and is useful for predicting the potential for anaerobic benzene degradation at other sites. The information is necessary and essential to design and implement successful bioremediation technology. The laboratory data obtained can be used to scale up and design full-scale bio-treatment system. The finding of *Betaproteobacteria* species dominant in the culture suggests the possibility of culturing the environmentally significant benzene-degrading microorganisms under denitrifying condition at PHC contaminated site, and its application on further sites bioremediation using bioaugmentation.

5.2. Future Work

During site selecting stage, initial survey of microbial activity in the soil and groundwater samples will reveal the presence and diversity of indigenous microbial community and their ability to degrade contaminates of interest. Further growth optimization for anaerobic benzene degradation is still needed, as the culture does not grow sufficiently significant over a short period of time. It is worthwhile to study the influence of parameters including compositions in the medium recipe on the growth of benzene-degrading cultures. The mechanisms through which toluene stimulates benzene degradation is yet unknown, and requires further investigation. The most convincing proof would be the finding of enzyme for methylation. Investigation of temporal and spatial changes in bacterial populations and the diversity of the microbial community during bioremediation process are recommended to offer a deep insight into the process. Further cultivation of *Betaproteobacteria* for degrading benzene anaerobically is suggested to confirm the finding in this study. Since microbial communities play a significant role in biogeochemical cycles, it is important to analyze the community structure and its changes during bioremediation process before bioaugmentation application. Extrapolation from laboratory scale to field scale may be subject to inaccuracies.

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APPENDIX

Chemical analysis for Recipe NP; Coates; Kazumi; SA, SA+T, SA+P; and BioTrap.

Recipe NP					
		Benzene (mg/L)			
	Days	Before	reading	After	Consumption
#1(soil sample + media)			1.15	1.14	
#2(soil sample + water + benzene)	1		25.45	25.19	
#3 (soil sample + media + benzene)			27.70	27.42	
#4 (soil sample + media + benzene)			25.66	25.40	
#5(sterilized soil sample + media + control)			21.60	21.38	
#1(soil sample + media)	8	1.14	0.90	0.90	0.24
#2(soil sample + water + benzene)		25.19	22.53	22.30	2.66
#3 (soil sample + media + benzene)		27.42	23.54	23.31	3.88
#4 (soil sample + media + benzene)		25.40	23.70	23.47	1.70
#5(sterilized soil sample + media + control)		21.38	21.42	21.21	-0.04
#1(soil sample + media)	15	0.90	0.70	0.70	0.20
#2(soil sample + water + benzene)		22.30	24.69	24.44	-2.38
#3 (soil sample + media + benzene)		23.31	26.02	25.77	-2.71
#4 (soil sample + media + benzene)		23.47	23.06	22.84	0.41
#5(sterilized soil sample + media + control)		21.21	22.63	22.41	-1.42
#1(soil sample + media)	22	0.70	0.60	0.59	0.10
#2(soil sample + water + benzene)		24.44	24.83	24.58	-0.38
#3 (soil sample + media + benzene)		25.77	24.66	24.41	1.11
#4 (soil sample + media + benzene)		22.84	21.93	21.72	0.90
#5(sterilized soil sample + media + control)		22.41	22.23	22.01	0.18
#1(soil sample + media)	29	0.59	0.50	0.50	0.09
#2(soil sample + water + benzene)		24.58	22.71	22.49	1.87
#3 (soil sample + media + benzene)		24.41	23.66	23.43	0.75
#4 (soil sample + media + benzene)		21.72	21.39	21.18	0.32
#5(sterilized soil sample + media + control)		22.01	21.06	20.85	0.95
#1(soil sample + media)	35	0.50	0.50	0.50	-0.01
#2(soil sample + water + benzene)		22.49	23.15	22.92	-0.66
#3 (soil sample + media + benzene)		23.43	23.51	23.28	-0.08
#4 (soil sample + media + benzene)		21.18	19.87	19.68	1.30
#5(sterilized soil sample + media + control)		20.85	19.68	19.48	1.17
#1(soil sample + media)	50	0.50	0.60	0.59	-0.10
#2(soil sample + water + benzene)		22.92	21.39	21.18	1.53
#3 (soil sample + media + benzene)		23.28	22.51	22.29	0.77
#4 (soil sample + media + benzene)		19.68	21.52	21.31	-1.85
#5(sterilized soil sample + media + control)		19.48	18.43	18.25	1.05

Recipe Coates					
		Benzene (mg/L)			
	Days	Before	reading	After	Consumption
#1(soil sample + media)			0.90	0.90	
#2(soil sample + water + benzene)	1		20.51	20.31	
#3 (soil sample + media + benzene)			18.68	18.51	
#4 (soil sample + media + benzene)			16.19	16.04	
#5(sterilized soil sample + media + control)			31.23	30.93	
#1(soil sample + media)	15	0.90	0.70	0.70	0.20
#2(soil sample + water + benzene)		20.31	18.25	18.15	2.06
#3 (soil sample + media + benzene)		18.51	18.48	18.24	0.03
#4 (soil sample + media + benzene)		16.04	15.98	15.68	0.06
#5(sterilized soil sample + media + control)		30.93	25.56	25.46	5.37
#1(soil sample + media)	29	0.70	0.65	0.60	0.05
#2(soil sample + water + benzene)		18.15	20.86	19.46	-2.71
#3 (soil sample + media + benzene)		18.24	16.46	16.25	1.78
#4 (soil sample + media + benzene)		15.68	14.71	14.57	0.97
#5(sterilized soil sample + media + control)		25.46	23.28	22.97	2.18

Recipe Kazumi					
		Benzene (mg/L)			
	days	Before	reading	After	Consumption
#1(soil sample + media)			5.29	5.24	
#2(soil sample + water + benzene)	1		26.52	26.26	
#3 (soil sample + media + benzene)			25.12	24.87	
#4 (soil sample + media + benzene)			26.93	26.66	
#5(sterilized soil sample + media + control)			30.66	30.36	
#1(soil sample + media)	15	5.24	5.47	5.42	-0.23
#2(soil sample + water + benzene)		26.26	23.02	22.80	3.23
#3 (soil sample + media + benzene)		24.87	24.98	24.73	-0.11
#4 (soil sample + media + benzene)		26.66	26.12	25.86	0.54
#5(sterilized soil sample + media + control)		30.36	26.04	25.78	4.32
#1(soil sample + media)	29	5.42	5.18	5.13	0.23
#2(soil sample + water + benzene)		22.80	22.86	22.63	-0.06
#3 (soil sample + media + benzene)		24.73	24.46	24.22	0.27
#4 (soil sample + media + benzene)		25.86	26.71	26.44	-0.85
#5(sterilized soil sample + media + control)		25.78	25.08	24.83	0.69

Recipe SA, SA+T, SA+P						
	Benzene Concentration (mg/L)					
Days	SA1	SA2	SAT1	SAT2	SAP1	SAP2
15	16.93	16.99	15.01	16.36	16.57	16.57
22	17.31	17.49	13.12	13.00	15.37	15.60
29	16.99	15.80	13.86	14.27	15.75	15.69
35	17.02	17.25	14.77	14.74	16.38	16.51
43	16.72	16.75	14.39	14.06	15.67	16.14
56	14.09	15.29	12.40	13.65	15.72	14.09
69	14.87	15.49	12.00	11.59	13.09	13.37
87	11.97	13.60	12.10	12.83	14.18	14.11
106	13.79	14.77	12.36	12.12	12.50	13.29
129	13.48	16.64	9.11	10.63	11.81	13.05
152	13.84	13.44	9.13	10.17	11.13	11.25
166	12.80	13.48	8.01	9.28	10.70	11.84
182	12.65	12.66		7.44	9.37	11.42
197	11.03	12.11		5.17	8.74	10.95
207	10.62	11.21		6.13	8.35	10.68
217	10.47	11.01		5.29	8.44	10.15
227	10.13	10.88		6.92	7.18	9.80

BioTrap		
Days	Benzene Concentration	Toluene Concentration
1	16.92	4.66
15	16.30	1.55
30	10.92	0.23
40	11.47	0.12
50	10.96	0.08
60	10.74	0.08